

# FUNCTION OF PSORIASIS SUSCEPTIBILITY GENE CCHCR1

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## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

- I Elomaa O, Majuri I, Suomela S, Asumalahti K, Jiao H, Mirzaei Z, Rozell B, Dahlman-Wright K, Pispä J, Kere J, Saarialho-Kere U, 2003: Transgenic mouse models support HCR as an effector gene in the PSORS1 locus. *Hum Mol Genet.* 13:1551-1561.
- II Tiala I, Suomela S, Huuhtanen J, Wakkinen J, Hölttä-Vuori M, Kainu K, Ranta S, Turpeinen U, Hämäläinen E, Jiao H, Karvonen SL, Ikonen E, Kere J, Saarialho-Kere U, Elomaa O, 2007: The CCHCR1 (HCR) gene is relevant for skin steroidogenesis and downregulated in cultured psoriatic keratinocytes. *J Mol Med.* 85:589-601.
- III Tiala I, Wakkinen J, Suomela S, Puolakkainen P, Tammi R, Forsberg S, Rollman O, Kainu K, Rozell B, Kere J, Saarialho-Kere U, Elomaa O, 2008: The PSORS1 locus gene CCHCR1 affects keratinocyte proliferation in transgenic mice. *Hum Mol Genet.* 17:1043-1051.

The publications are referred to in the text by their roman numerals.

## ABBREVIATIONS

AP-1	Activator protein 1
APC	Antigen-presenting cell
ATF	Activating transcription factor
BrdU	Bromodeoxyuridine
CCHCR1	Coiled-Coil $\alpha$ -Helical Rod Protein 1
CDSN	Corneodesmosin
DC	Dendritic cell
EGF	Epidermal growth factor
HLA	Human leukocyte antigen
IL	Interleukin
K14	Keratin 14
mRNA	Messenger ribonucleic acid
PSORS	Psoriasis susceptibility locus
RBP3	RNA polymerase II subunit C
SCID	Severe combined immunodeficiency
StAR	Steroidogenic acute regulatory protein
STAT	Signal transducer and activator of transcription
TPA	12-O-tetradecanoylphorbol-13acetate
VEGF	Vascular endothelial growth factor
VDR	Vitamin D receptor

## ABSTRACT

Psoriasis is a chronic skin disease characterized by abnormal keratinocyte proliferation and differentiation, neoangiogenesis and inflammation. Its etiology is multifactorial, as both the environmental and genetic factors have an important role in the pathogenesis of psoriasis. The exact disease mechanism behind psoriasis still remains unknown. The most important genetic susceptibility region for psoriasis has been located to PSORS1 locus in chromosome 6. The area includes multiply good candidate genes but the strong linkage disequilibrium between them has made genetic studies difficult. One of the candidate genes in PSORS1 is CCHCR1, which has a psoriasis-associated gene form CCHCR1\*WWCC. The aim of the study was to elucidate the function of CCHCR1 and its potential role in the pathogenesis of psoriasis.

In this study, transgenic mice expressing either the healthy or psoriasis-associated gene form of CCHCR1 were engineered and characterized. Mice were phenotypically normal but their gene expression profiles revealed many similarities to that observed in human psoriatic skin. In addition, the psoriasis-associated gene form had specific impacts on the expression of many genes relevant to the pathogenesis of psoriasis. We also challenged the skin of CCHCR1 transgenic mice with wounding or 12-O-tetradecanoylphorbol-13-acetate (TPA). The experiments revealed that CCHCR1 impacts on keratinocyte proliferation by limiting it. In addition, we demonstrated that CCHCR1 has a role in steroidogenesis and showed that both CCHCR1 forms promote synthesis of steroids. Also many agents relevant either for steroidogenesis or cell proliferation were shown to regulate the expression level of CCHCR1.

The present study showed that CCHCR1 has functional properties relevant in the context of psoriasis. Firstly, CCHCR1 affects proliferation of keratinocytes as it may function as a negative regulator of keratinocyte proliferation. Secondly, CCHCR1 also has a role in steroidogenesis, a function relevant both in the pathogenesis of psoriasis and regulation of cell proliferation. This study suggests that aberrant function of CCHCR1 may lead to abnormal keratinocyte proliferation which is a key feature of psoriatic epidermis.

## INTRODUCTION

Psoriasis is a chronic inflammatory skin disease affecting 1-3% of Caucasians. Psoriasis is characterized by red well-defined skin plaques which are typically located on the scalp, knees or elbows. The molecular level pathogenesis of psoriasis is still poorly understood. The main histological features observed in psoriatic skin are the hyperproliferation and impaired differentiation of keratinocytes, infiltration of inflammatory cells and vascular changes. The disease lessens the quality of life and in addition, it also predisposes to other chronic diseases, including type II diabetes and cardiovascular diseases. There is no curative treatment available for psoriasis and the current psoriasis treatments may cause significant side effects.

Psoriasis is a multifactorial disease, as both environmental and genetic factors are needed for disease onset. The major susceptibility locus for psoriasis, PSORS1, is located in the HLA-region on chromosome 6. PSORS1 includes multiple genes, of which three, HLA-C, corneodesmosin and Coiled-Coil  $\alpha$ -Helical Rod Protein 1, may be considered candidate genes for psoriasis. Strong linkage disequilibrium between the candidate genes has hindered the genetic studies aiming to identify the PSORS1 effector gene. Coiled-Coil  $\alpha$ -Helical Rod Protein 1, also called CCHCR1, has altered expression in psoriatic skin and a psoriasis-associated risk allele CCHCR1\*WWCC.

The aims of this study were to investigate the biological role of CCHCR1 and its relevance in the pathogenesis of psoriasis using functional studies with mouse models and cultured keratinocytes. An important element of the study was the transgenic mice expressing either wild-type CCHCR1 or psoriasis-associated CCHCR1\*WWCC form in their epidermis. In addition, the role of CCHCR1 as StAR-binding protein in steroidogenesis was elucidated. The functional studies of CCHCR1 support its role as an important PSORS1 effector gene.



## REVIEW OF THE LITERATURE

### 1. Clinical features of psoriasis

Psoriasis is a chronic relapsing skin disease with wide variations in morphology of clinical lesions and course (Naldi&Gambini 2007). Characteristic for psoriasis are the red, well demarcated skin plaques, which vary in size and are covered by sheer scales (Lomholt 1963). Both the environmental factors and suitable genetic background are needed for disease onset, making psoriasis an example of a multifactorial disease.

#### 1.1 Clinical subtypes of psoriasis

*Psoriasis vulgaris* (PV) is the most common type of psoriasis, accounting for 90 % of psoriasis cases. In PV the plaques are red or pink, diverse in size and form and well-outlined from the surrounding normal skin. Plaques are usually symmetrically distributed, located commonly on the extensor aspects of elbows and knees, and on the scalp (Lomholt 1963). As PV includes different site-specific variants and forms, it is most probable that PV will turn out to be several closely-related, but still discrete, disease conditions with different phenotypic and genotypic characteristics. Different subtypes could explain the variability in the patients' responses to therapy, as is the case especially with the T lymphocyte-targeted biological agents (Griffiths&Barker 2007).

Another form of psoriasis is guttate psoriasis (GP). GP is characterized by small plaques over the upper trunk and proximal extremities. The plaques often emerge after a  $\beta$ -hemolytic streptococcal infection or viral infection. GP is most commonly seen in children or adolescents and is self-limiting, usually resolving within 3 to 4 months. However, a substantial portion of individuals with guttate psoriasis develop *psoriasis vulgaris* later in their lives (Naldi et al. 2001).

There are also other psoriasis subtypes, namely inverse, erythrodermic and pustular psoriasis. In addition also palmoplantar pustulosis (PPP), characterized by sterile pustules on the palms and soles, is still often described as a subtype of psoriasis. Although 25% of the PPP patients also have chronic plaque psoriasis, PPP has specific features when compared to PV. Although PV is equally common among men and women, PPP patients

are predominantly women. Also studies implicate that the genetic background behind PPP and PV is different (Asumalahti et al. 2003). Rather than being a form of psoriasis, PPP should be considered to show co-morbidity with PV.

### *1.2 Prevalence*

Psoriasis is found world-wide and its prevalence varies from 0 % to 11.8 % in different populations and ethnic groups (Gudjonsson&Elder 2007). The incidence is the highest among Caucasians and lowest among those of Japanese and African descent. Denmark and the Faroe Islands have the highest psoriasis prevalence in Europe, 2.9%, as the average psoriasis prevalence among Europeans, as well as Finns, is around 2% and 2.9% (Lomholt 1963; Brandrup&Green 1981; Brandrup et al. 1982; Gudjonsson&Elder 2007). In the United States 2.2-2.6% of the population is affected with psoriasis. The prevalence varies between the ethnic groups in the US, as the incidence of psoriasis among African Americans is only 1.3%. This is in congruence with the fact that the prevalence of psoriasis among West Africans is only 0.3% and the ancestors of African Americans mostly originated from that area. There is also considerable difference between West and East African populations, as the psoriasis prevalence is almost 7 times higher in the eastern population (Gudjonsson&Elder 2007). The prevalence of psoriasis among Asian populations is rather low, only 0.3% (Yip 1984). Some populations, namely American Samoas and South American Indians, lack psoriasis completely (Gudjonsson&Elder 2007). Psoriasis incidence has increased significantly during the last three decades, but the reason behind this rise is still unknown (Icen et al. 2009). Latitude also affects psoriasis prevalence, probably through the beneficial effects of sunlight on psoriasis. Psoriasis affects men and women as often, even though there have been studies showing differences between the sexes (Braathen et al. 1989; Christophers 2001; Icen et al. 2009).

### *1.3 Type I and II psoriasis*

Psoriasis has two subclasses based on age of onset and the presence of affected family members. The early onset group (type I) develops psoriasis before age 40, women slightly earlier than men. The mean age of onset is 33 years and 75% of the cases occur before age 46 (Nevitt&Hutchinson 1996). In the late onset group (type II), the disease erupts substantially later, typically at the age of 57-60 years (Henseler&Christophers 1985; Smith et al. 1993). Subgroups differ also in the presence of the familial component. In the early

onset group, nearly half of the patients have an affected parent, whereas in the late onset group there is usually no family history of psoriasis present (Henseler&Christophers 1985). There are also different patterns in the HLA association: early onset psoriasis shows strong association with HLA-Cw6, whereas the late onset form has increased frequency of HLA-Cw2 and HLA-B27 (Henseler 1997). It has indeed been shown that increasing age of onset correlates with diminishing association with HLA-Cw6 on chromosome 6 (Allen et al. 2005). There are also differences in the clinical picture between the groups, as the early onset group has more a unstable and severe disease course when compared to the late onset type (Lomholt 1963; Stuart et al. 2002).

#### *1.4 Pathogenesis of psoriasis*

##### *1.4.1 Epidermis*

Skin is composed of two layers, the outer epidermis and the inner dermis. The epidermal layer is composed of 10-20 layers of keratinocytes. Epidermal keratinocytes can be divided into four different layers according to the level of differentiation (Figure 1). The innermost layer, the basal layer (*stratum basale*) includes stem cells, which give rise to transiently amplifying keratinocytes. In the spinous layer (*stratum spinosum*), keratinocytes are connected by desmosomes and go through early differentiation, after which they will undergo apoptosis. In the granular layer (*stratum granulosum*) keratinocytes go through late and terminal differentiation and also lose their nuclei during this phase. Keratohyalin granules are found abundantly in the granular cells. In the outermost horny layer, keratinocytes die and form *stratum corneum* (Nemes&Steinert 1999). In healthy skin, the maturation of keratinocytes through the phases above usually takes 52-75 days. Keratinocytes are the most prevalent cell type in the epidermis, comprising about 95 %. Also pigment-producing melanocytes, Lagerhans cells participating in antigen recognition, and receptors of touch, neuroendocrine Merkel cells, are found in the epidermis. Under the epidermis lies the connective tissue layer, the dermis, where blood vessels are also located (Rao et al. 1996).

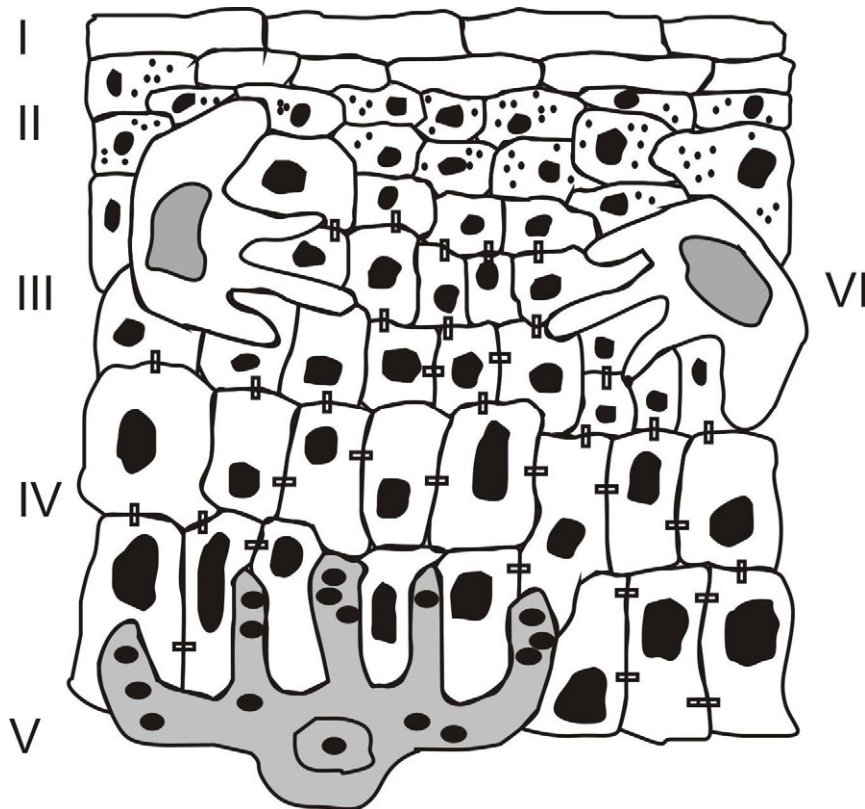


Figure 1. Structure of epidermis. Keratinocytes form four layers based on level of differentiation (I=*Stratum Corneum*, II=*Stratum Granulosum*, III=*Stratum Spinosum*, IV=*Stratum Basale*). In addition to keratinocytes, other cell types found include melanocytes (V), Merkel cells and Langerhans cells (VI).

#### 1.4.2 Psoriatic plaques

As the disease course in psoriasis waxes and wanes, the histopathological picture of the plaques (Figure 2) also varies according to the age of the lesions. In the early stages changes in the dermis dominate. These changes include minor superficial perivascular T-lymphocyte infiltrate, followed by development of dilated blood vessels within dermal papillae and mild dermal edema. Also minor spongiosis with rare T lymphocyte and/or neutrophil extension into the epidermis can be observed in the earliest stage of psoriasis plaque. Intraepidermal T lymphocytes are predominantly CD8 positive. As the plaque develops, slight hyperplasia typical for psoriatic lesions is observed in the epidermis. The amount of neutrophils in the epidermis increases and parakeratosis also emerges. In the fully developed plaque, epidermal hyperplasia is notable, showing several typical features, including regular elongation of rete ridges with characteristic enlargement in their tips, reciprocal elongation of intervening dermal papillae with dilated and tortuous capillaries

and thinning of the epidermis above dermal papillae. Hyperkeratosis in the epidermis is prominent and its structure, with the alternating orthokeratosis and parakeratosis, suggests fluctuation in the epidermal growth activity in the lesion area. Vascular changes, namely increased vascularity, are also observed in the psoriatic plaques. Blood vessels in the dermal papillae are hyperplastic and hyperpermeable and show increased expression of E-selectin, ICAM-1 and VCAM-1, in addition to elevated VEGF and VEGFR receptor expression (Hvid et al. 2008). Neoangiogenesis can also be observed (Longo et al. 2002). Interestingly, keratinocytes produce angiogenic factors that promote abnormal dermal vascular proliferation and angiogenesis (Griffiths&Barker 2007). Plaques resolving or treated go through progressive reduction of parakeratosis and the number of neutrophils in the stratum corneum also decrease. The granular zone reforms and keratinocytes become orthokeratotic. Hyperplastic changes of the epidermis resolve later and also vascular changes recover slower (Murphy et al. 2007).

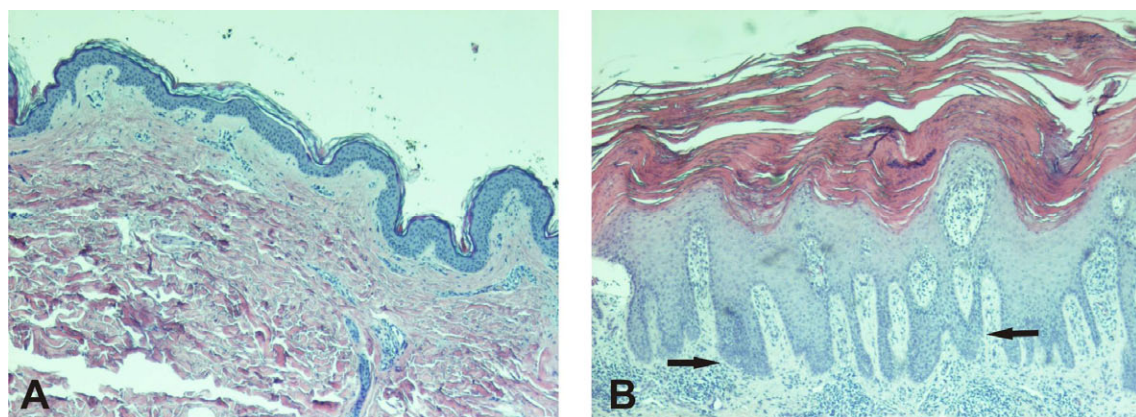


Figure 2: Histology of healthy (A) and psoriatic (B) skin. Psoriatic skin shows epidermal acanthosis, elongation of rete ridges (indicated by arrows) with reciprocal elongation of intervening dermal papillae and inflammatory infiltrate (40X magnification).

Psoriasis also causes alterations in epidermal keratin expression. The keratin expression in the basal layer is similar between the healthy and psoriatic skin as they both express K5 and K14 keratins. In healthy skin suprabasal keratinocytes express differentiation specific keratins 1 and 10, but in psoriatic suprabasal keratinocytes their expression is downregulated and hyperproliferative keratins 6 and 16 are expressed instead. In addition, keratin 17 expression is abundant in psoriatic lesions (Bonnekoh et al. 1995; Leigh et al. 1995; Rao et al. 1996).

An interesting aspect of the plaques is the chemical shield they offer. Psoriatic patients are quite resistant to infectious organisms and it has been argued that the compensatory response of immune system cells, together with abundant expression of genes responsible for the innate immunity, lie behind this divergent phenomenon (Buchau&Gallo 2007). Another interesting property of psoriatic plaques is that they only rarely associate with skin cancer (Nickoloff 2001).

### *1.5 Features behind psoriasis*

Psoriasis is referred to as an autoimmune disease. This is based on the chronic inflammation and the absence of a pathogen or other foreign antigens causing the inflammation. Psoriasis is considered to be caused by a combination of genetic, immunological and environmental factors. Different kinds of environmental factors are known either to trigger the disease or to worsen its course. These include, for example, both physical and psychological stress, excessive alcohol intake and several drugs, such as lithium and beta-blockers (Dika et al. 2007). The formation of psoriasis plaque in response to cutaneous trauma, for example trauma from scratching or sunburn, is known as Koebner's phenomenon (Raychaudhuri et al. 2003).

However, there is ongoing debate about the initial factors causing the disease and differing views are constantly coming forward. The question involving the initial factor or cell type behind the disease has focused mainly on the possible roles of immune system cells or keratinocytes in the initiation of pathogenesis. There is a convincing amount of data supporting both points of view. Aberrant keratinocyte metabolism as a primary cause behind the disease is based, among other things, on increased keratinocyte proliferation and an altered keratin expression profile in psoriatic lesions. The role of keratinocytes is also promoted by the fact that alterations in the expressions of intracellular signaling molecules exclusively in basal keratinocytes are capable of inducing skin inflammation similar to that observed in psoriasis. This observation is based on the Jun-B/c-jun double mutant mouse and corneodesmosin mouse (see section "Genetically engineered mouse models"), which develop skin disease resembling psoriasis (Zenz et al. 2005; Matsumoto et al. 2008). Most models seem anyhow to promote the idea that psoriasis is caused by abnormalities in the interaction between both keratinocytes and immune system cells, not in either of them alone (Ghoreschi et al. 2007). T lymphocytes are found in psoriatic lesions and T cell-derived cytokines are also abundant in psoriatic skin, as well as numerous antigen-

presenting cells producing inflammatory cytokines and chemokines (Buchau&Gallo 2007). Drugs modifying T lymphocyte functions have also been successfully used as psoriasis treatment. Furthermore, bone marrow transplantation can affect psoriasis status: when a psoriatic patient has received a transplant from a healthy person, psoriatic lesions have healed. The phenomenon also occurs vice versa (Eedy et al. 1990; Gardembas-Pain et al. 1990; Kanamori et al. 2002). In addition, the evidence gained with the immunodeficient SCID mouse model highlights the role of immunomechanisms in the pathogenesis of psoriasis: mice with grafted non-lesional skin from a psoriatic person develop psoriasis-like alterations to the transplanted skin after injection of autologous immunocytes. When the transplanted skin originated from a healthy person similar impact did not occur (Wrone-Smith&Nickoloff 1996). The necessity of T lymphocytes can be questioned based on the Rag2-deficient Jun-B/c-jun double mutant mice. Mice develop a psoriasis-like condition with epidermal thickening, altered keratinocyte maturation and vascular changes in the absence of B and T cells (Zenz et al. 2005). In the absence of full mechanistic explanations more research is needed to conclude which cell type initiates pathogenesis of psoriasis.

In addition, it is inadequate to consider psoriasis only as a disease of the skin as in fact, psoriasis is associated with many systemic disorders like Crohn's disease, metabolic syndrome, type 2 diabetes and depression. The disease risk can also vary according to the severity of psoriasis. This is seen with psoriasis and cardiovascular disease: patients with mild psoriasis do not have increased risk, but if psoriasis is moderate or severe the relative risk for cardiovascular disease is almost three-fold. In addition, psoriasis associates with cancers such as lymphoma, but it remains unsolved whether the relation is with the disease itself or with the treatments used like photochemotherapy (Griffiths&Barker 2007).

### 1.5.1 Keratinocytes

Keratinocytes have a significant role in the formation of psoriasis plaque. Various types of alterations can be seen in the properties of keratinocytes in the plaques when compared to keratinocytes in healthy epidermis. Proliferation of keratinocytes is raised almost 50-fold but the factors causing the increase are still unknown (Sabat et al. 2007). There are two mechanisms that facilitate the increased proliferation. The cell cycle is considerably shorter in psoriatic keratinocytes compared to healthy keratinocytes (Ortonne 1999). In addition, the cell population participating in the accelerated cell cycle is greater in psoriatic

epidermis than that in healthy skin (Weinstein et al. 1985). Two possible sources for the increased dividing cell population have been proposed: the increased number of stem cells participating in cell divisions or the larger number of cell cycles that transiently amplifying cells go through before entering apoptosis. In addition to impaired proliferation, keratinocyte differentiation is also dysfunctional in psoriatic skin. Impaired differentiation results in parakeratosis and loss of the granular layer. The role of the keratinocytes in psoriasis is beyond doubt but the molecular mechanisms behind the alterations are still poorly understood.

### 1.5.2 Inflammatory cells

Inflammatory cells including T lymphocytes, neutrophils, mast cells, macrophages and dendritic cells are found in psoriasis plaques. The antigens or auto-antigens responsible for the inflammatory reaction have not been identified despite the vast amount of research. A self-peptide cross-reacting with streptococci is one of the candidates as streptococcal infections precede 90% of psoriasis type I cases (Ghoreschi et al. 2007). Dendritic cells (DC) act as antigen-presenting cells (APC) to initiate the immunoresponse after stimulation by an as yet unknown signal. Activated DCs migrate to lymphatic tissue and secrete chemokines to attract naïve T lymphocytes, which are then activated and differentiated to Th1 and Th17 type cells. Movement of the activated T lymphocytes from the periphery to the skin is elementary for the plaque to develop. The invasion is based on tissue-specific ligand receptor interaction and on that account skin homing T cells produce L-selectin, LFA-1 (lymphocyte function associated antigen 1) and CLA (cutaneous lymphocyte antigen) adhesion molecules. Resident skin cells also express molecules important in skin homing, including ICAM-1 (intercellular adhesion molecule 1) expressed by epidermal keratinocytes and E- and P-selectins in dermal capillaries. The initial interaction is formed between the L-selectin of T lymphocytes and E- and P-selectins expressed by vascular endothelial cells, after which other ligand-receptor interactions also emerge to continue the inflammation reaction. Both CD4 and CD8 positive T lymphocytes are in psoriasis plaque, CD8<sup>+</sup> cells predominantly within the epidermis and CD4<sup>+</sup> cells within the dermis. Especially the role of CD4<sup>+</sup> cells is critical in the pathogenesis of psoriasis. Other inflammatory cells also take part in pathogenesis but their role is not as well understood. Both macrophages and mast cells produce cytokines including tumor necrosis factor (TNF),



interferon  $\gamma$  (IFN- $\gamma$ ) and interleukin 8 (IL-8), which are essential for psoriasis (Ghoreschi et al. 2007; Griffiths&Barker 2007; Nickoloff et al. 2007).

### 1.5.3 Cytokines

Immunocytes in skin communicate with other cells via production of cytokines and chemokines. Cytokines can influence various processes including cell proliferation, differentiation and inflammatory and anti-inflammatory reactions. In the pathogenesis of psoriasis, several cytokines form a complex network and it is unlikely that perturbations in the function of one of them would have causative effects on the disease course (Bonifati&Ameglio 1999). Nevertheless, it has been suggested that there are cytokines of primary and secondary importance when considering the impact of a single cytokine in the pathogenesis (Nickoloff et al. 2007). A substantial number of cytokines have been implicated in psoriasis. Psoriasis is commonly considered to be a Th1 type disease and cytokines of the pathway, including IL-2, IL-12, IFN- $\gamma$  and TNF- $\alpha$ , are abundantly found in psoriasis plaques (Uyemura et al. 1993; Schlaak et al. 1994; Austin et al. 1999). Interleukin-2 is a T cell growth factor, which promotes T cell function, as well as stimulates natural killer cell activity, and promotes production of a wide variety of cytokines (Kemmett et al. 1990; Bonifati&Ameglio 1999; Gaspari 2006). Interferon  $\gamma$  is an important immunoregulator and also has antiproliferative properties. Furthermore it can induce the expression of ICAM-1 in keratinocytes and endothelial cells and thus influence invasion of skin homing T lymphocytes in psoriatic skin (Griffiths et al. 1989). Tumor necrosis factor  $\alpha$  is produced by keratinocytes and has a focal role in skin inflammatory processes (Pietrzak et al. 2008). Proinflammatory cytokines IL-6 and IL-8, capable of promoting keratinocyte proliferation, are also expressed abundantly in psoriatic skin (Grossman et al. 1989; Tuschil et al. 1992). Recent additions to the list of cytokines relevant in psoriasis are IL-12 and IL-23. In psoriatic skin IL-23 is overproduced by DCs and keratinocytes and promotes Th 17 type T cells' survival and the production of IL-17 and IL-22, which are also considered to be significant in psoriasis (Fitch et al. 2007; Nickoloff et al. 2007). Interleukin 17 promotes accumulation of neutrophils, dendritic cells and T cells and also has an impact on barrier function. Interleukin-22 triggers keratinocyte hyperproliferation and downregulates genes associated with keratinocyte differentiation, which are functional aspects relevant for the pathogenesis of psoriasis (Chen et al. 2003; Nograles et al. 2008). Interleukin-12 participates in several processes relevant for psoriasis including cell proliferation and

angiogenesis. It also promotes T lymphocyte activation and differentiation by promoting the Th1 pathway (Pietrzak et al. 2008). In addition to the aforementioned cytokines, other cytokines as well as chemokines are found and may have an impact on the pathogenesis of psoriasis.

### *1.6 Current view of the psoriasis disease model*

According to Sabat *et al.*, the development of psoriatic plaque begins with antigen recognition and uptake by antigen-presenting cells (APC) (Sabat et al. 2007). In psoriasis the dendritic cells (DC) are the most common type of antigen-presenting cells. Antigens are presented on the DC surface usually as MHC class II molecules, which can be recognized by T cell receptor of CD4<sup>+</sup> T cells. In addition DCs can present antigens on MHC class I molecules, which lead to the activation of CD8<sup>+</sup> T cells. DCs also enhance the production of adhesion and co-stimulatory molecules to facilitate its interaction with T cells. The antigens behind the process are not known (Sabat et al. 2007).

Naive T cells have to go through maturation in lymphatic tissues. CD4<sup>+</sup> T cells have a strong affinity to MHC II-peptide complex and they stick together by forming an immunological synapsis. Interaction between ICAM-1, produced by DCs, and LFA-1, from T cells, is one of the most important in order to facilitate the DC-T cell interaction. Naive T cells can mature either to Th1, Th2, Th17 or regulatory T cells and subtype is guided by the selection of a soluble mediator present during the activation of naive T cells. After the activation, T cells express cutaneous lymphocyte-associated antigen (CLA), which directs T cells to inflamed skin. P- and E-selectins expressed by endothelial cells are also important for T cell skin homing (Sabat et al. 2007).

In inflamed skin, T cells enter the tissue and participate in the inflammation reaction. Interaction between P- and E- selectins and CLA, as well as other selectin ligands, facilitate leukocytes rolling along the blood vessel wall as they decrease the rolling velocity. Interestingly expressions of P- and E-selectins are upregulated in psoriatic skin, possibly stiffening the inflammation observed in psoriatic plaque. T cells recognize chemokines secreted by the endothelial cells, become active and a tight adhesion, facilitated by integrins produced by T cells and their ligands expressed by endothelial cells, is formed between the cells. The most important molecule in skin homing is LFA-1 binding to ICAM-2. T cells

probably enter the endothelial wall through pores formed between endothelial cells in an integrin-dependent process called diapedesis (Sabat et al. 2007).

In the skin T cells are reactivated by different kinds of APCs, which also include keratinocytes. Interferon- $\alpha$  (IFN- $\alpha$ ), produced by APCs, has an important role in T cell reactivation and proliferation. Interestingly, T cells in psoriatic skin have prolonged and increased IFN- $\alpha$  response when compared to healthy skin. Interferon- $\alpha$  enhances INF- $\gamma$  expression in T cells. Also cytokines produced by APCs, especially IL-23 and IL-6, have an important role in reactivation. After reactivation T cells also express a variety of cytokines. T cell response leads to the activation of keratinocytes and the activation is carried out by Th17 and different cytokines produced by macrophages, DCs and keratinocytes themselves. Keratinocyte activation leads to their increased proliferation and alterations in the maturation process. In addition activated keratinocytes produce a vast variety of mediators, which further promote immigration of inflammatory cells and induce angiogenesis, thus enhancing phenomena relevant for the pathogenesis of psoriasis (Krueger, 2002; Sabat et al. 2007).

### *1.7 Psoriasis treatments*

Psoriasis treatments include therapy with various topical agents, phototherapy and systemic treatments including biological agents. The selection of appropriate treatment depends on the severity and localization of the skin symptoms and the benefits and risks of different treatment options. Emotional impact of the disease is taken into consideration as well (Feldman et al. 2008).

The major psoriasis treatments include topical corticosteroid and calcipotriol applications, photochemotherapy with psoralens and ultraviolet A (PUVA), phototherapy with ultraviolet B and systemic treatment with acitretin, methotrexate or cyclosporine. These treatments have a wide variety of well-known side effects such as fast relapse times, development of tolerance, sunburn reactions, impaired renal or liver function, teratogenicity and bone marrow suppression (Linden&Weinstein 1999). Psoriasis treatments affect T cell function, for example PUVA depletes lymphocytes, corticosteroids have immunosuppressive properties and cyclosporine has a major inhibitory effect on T cell activation. In addition cyclosporine has a strong antiproliferative effect on keratinocytes. Methotrexate also has a

similar kind of dual action, both in suppression of lymphocytes as well as in keratinocyte proliferation (Linden&Weinstein 1999; Krueger 2002). Newly developed biological psoriasis therapeutics are also targeted to T cell functions. They are a promising alternative to psoriasis treatment, with relatively good action and possibly milder side effects in some patients (Krueger 2002). One of the newly developed biological treatments is tumor necrosis factor (TNF)-blocking monoclonal antibodies (Rozenblit&Lebwohl 2009). Long term studies are still needed to ensure their efficacy and safety, but based on early studies antibody treatments blocking TNF seem to have great benefits. Despite the wide selection of psoriasis treatments, there is no curative treatment available for psoriasis at the moment (Linden&Weinstein 1999).

## **2. Mouse models for psoriasis**

Animal models are highly important in the research of disease conditions. They facilitate a detailed study of the profound molecular mechanisms behind diseases and they are also used in the development of treatments. There are an extensive amount of aspects to take into account when thinking over the properties of a good animal model. To be a good model for psoriasis, the mouse model should manifest the key histological features observed in human psoriasis as well as the typical immunological reactions. A good model should also have a response to therapeutic agents similar to psoriatic patients (Gudjonsson&Elder 2007; Danilenko 2008). In addition to humans, only rhesus monkeys develop chronic dermatitis resembling psoriasis both clinically and histopathologically (Lowe et al. 1981). Similar conditions have also been observed in other monkey species, dogs and pigs, but in these the occurrence is only sporadic (Boehncke&Schon 2007).

There are many challenges in the development of a psoriasis mouse model because of the profound differences between human and mouse skin (Figure 3). These include the considerably difference in the size of the organism and fur covered skin of mouse with densely spread hair follicles as human skin is composed mostly of interfollicular areas. Human and mouse hair follicles differ also in gene expression profiles. In human skin there are differences in the gene expression observed in outer shoot sheath of the hair follicles and interfollicular areas as there is no similar phenomenon found in mouse skin. Interfollicular areas in mouse also lack rete ridges. In addition mouse skin is 75 % thinner than human skin and it is composed of only 2-3 keratinocyte layers. There are also

differences in the non-epithelial tissues. Human skin has a thicker dermis and only small cutaneous muscle rudiments in the face and neck whereas mice have a continuous cutaneous muscle layer. Mouse skin also recovers without scarring, indicating differences in wound healing processes. In addition many immunological aspects vary between human and mouse, mouse-specific dendritic and T cell subtypes as an example. Mice and humans also show differences in their genetic background since humans are outbred and laboratory mice are mainly inbred (Gudjonsson et al. 2007).

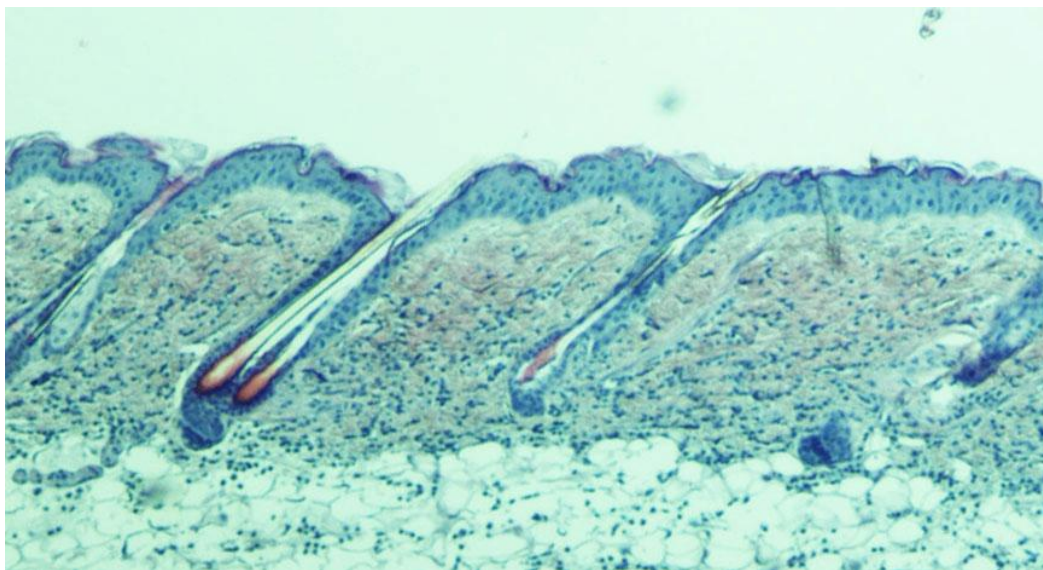


Figure 3: Histology of mouse skin. Mouse skin has densely spread hair follicles, interfollicular areas have no rete ridges and the epidermis is composed of 2-3 keratinocyte layers (40X magnification).

Absence of a suitable animal model has caused a great disadvantage for psoriasis research. However, various types of mouse models have been identified and developed, some showing more similarities to human psoriasis than others. It is notable that most of the mice presenting psoriasis-like features have not been developed in order to produce psoriasis mouse models but instead their psoriasis-like phenotype has evolved as a response to modifications done for other purposes. Mouse models can be divided into three groups: spontaneous models, xenograft models and genetically engineered models (Table 1).

Table 1. Summary of various psoriasis mouse models and their characteristics relevant for the pathogenesis of psoriasis.

Model	Acan- thosis	Altered epidermal differentiation	Increased vasculari- zation	Epidermal T cell infiltration	Reference
<b>Spontaneous models</b>					
Chronic proliferative dermatitis	Yes	Focal parakeratosis	Yes	Yes	HogenEsch et al. 1993; HogenEsch et al. 2001; Kim et al. 2002
Flaky skin	Yes	Focal parakeratosis	Yes	Yes	Sundberg et al. 1997
<b>Genetically modified models</b>					
<i>Targeted to immune system</i>					
K14-IL20 transgenic	Yes	Yes	No	No	Blumberg et al. 2001
K14-IL6 transgenic	Yes	No	No	No	Turksen et al. 1992
K14-p40 transgenic	Yes	No	Yes	Yes	Kopp et al. 2001; Kopp et al. 2003
<i>Targeted to vascular endothelium</i>					
K14-VEGF transgenic	Yes	Epidermal expression of hypoproliferative keratins	Yes	Unknown	Detmar et al. 1998; Xia et al. 2003
Tie 2 transgenic	Yes	Yes	Yes	Yes	Voskas et al. 2005; Voskas et al. 2008
<i>Targeted to epidermal keratinocytes</i>					
CDSN deletion	Yes	Yes	Yes	Yes	Matsumoto et al. 2008; Zenz et al. 2005
JunB/c inducible epidermal deletion	Yes	Parakeratosis: upregulation of S100A8 and S100A9	Yes	Yes	Zenz et al. 2005
K14-KGF transgenic	Yes	Epidermal expression of hypoproliferative keratins	Yes	Yes	Guo et al. 1993; Sano et al. 2005a
K5-Stat3 transgenic	Yes	Epidermal expression of hypoproliferative keratins	Yes	No	Sano et al. 2005a
K14-TGF $\alpha$ -transgenic	Yes	Parakeratosis	Unknown	In some animals	Vassar&Fuchs 1991
<b>Xenotransplantation models</b>					
SCID transplantation	Yes	Epidermal expression of hypoproliferative keratins	Yes	Yes	Boyman et al. 2004
AGR129 transplantation	Yes	Epidermal expression of hypoproliferative keratins	Yes	Yes	Bhagavathula et al. 2005; Voskas et al. 2005; Xia et al. 2003

## 2.1 Spontaneous mouse models

The first potential animal models for psoriasis were mice with spontaneous mutations causing a psoriasis-form phenotype. Probably the best of the spontaneous models is “the flaky skin mouse” ( $Ttc^{fsn}/Ttc^{fsn}$ ), which shows enhanced proliferation and hyperkeratosis of stratified squamous epithelia, positive Koebner’s phenomenon after tape stripping, intraepidermal invasion of neutrophils and an increased amount of epidermal growth factor receptors. However the pathogenesis behind these phenomena is unknown and the flaky skin mice do not express all the psoriatic features as both T cell infiltration and expression of hyperproliferative keratins is missing. Mice also have additional health problems independent from psoriasis. Another spontaneous model is the chronic proliferative dermatitis mouse ( $Sharnin^{cpdm}/Sharnin^{cpdm}$ ). It develops eosinophilic inflammation in various tissues, including skin, leading to acanthosis. Deficiency of this model is the Th2-driven inflammation, whereas inflammation in human psoriasis is Th1 and Th17-mediated. There are also other spontaneous mutations causing psoriasis-like phenotypes in mice. These animals often show characteristics typical for psoriatic dermis and epidermis. However, they generally lack T cell involvement, which is considered to be central in the pathogenesis of psoriasis (Gudjonsson et al. 2007; Danilenko 2008).

## 2.2 Xenograft mouse models

In xenograft models foreign skin is transplanted to an immunodeficient mouse. Transplanted skin can originate from a psoriatic patient and can be either non-lesional or lesional skin. Xenograft models are among the best animal models for psoriasis available at the moment as they can incorporate the genetic, phenotypical and immunopathological processes all relevant in psoriasis and are also considered to be the most faithful to human conditions. Xenograft models also have limitations: they are rather demanding to execute technically and also the supply of human psoriatic skin is problematic (Gudjonsson et al. 2007; Danilenko 2008).

A recent example of a xenograft model used in psoriasis research is the study of Stenderups *et al.* which showed that IL-20 has a critical role in the pathogenesis of psoriasis. In the study both plaque and non-lesional psoriatic skin samples were grafted on immunodeficient mice and the mice were treated either with recombinant human IL-20 or anti-IL-20

antibodies. They showed that blocking the IL-20 signaling induces resolution of psoriasis and also inhibits the onset of psoriasis. They also showed that IL-20 infusion, in addition to injection of non-activated leukocytes, promotes plaque formation in psoriatic non-lesional skin grafts (Stenderup et al. 2009).

### 2.2.1 The nude mouse

One of the xenograft models is the nude mouse, a mouse with a deficient thymus causing the incapability to produce mature T cells and resulting in impaired T cell function. The thymus deficiency, as well as disruption of normal hair growth, is caused by a mutation in FOXP1 (Forkhead box P1, aka WHN, winged helix nude) on chromosome 11 (Nehls et al. 1994). It has been shown with the nude mouse that psoriatic features of a transplanted psoriatic plaque can be maintained even longer than two months without the presence of functional T cells (Boehncke&Schon 2007; Gudjonsson et al. 2007).

### 2.2.2 The SCID mouse

Another xenograft mouse model is the SCID mouse, which lacks both humoral and cellular immunity because of a mutation in a DNA-dependent protein kinase enzyme. The mutation causes defects in the antigen receptor gene rearrangements in lymphocytes and thus has an essential role in T and B cell development, causing severe combined immunodeficiency (SCID). The disadvantage of the SCID model is the variability of immunological properties between mouse lines with different genetical backgrounds. The phenotype is also reversible, reversibility depends on the mouse strain used and the age of the mice. Another disadvantage of the SCID mouse is the degradation of injected single cell suspensions. Because of this degradation this model is not well suited for studies including T cell engraftments. However, skin grafts are well preserved in SCID mouse (Boehncke&Schon 2007; Gudjonsson et al. 2007).

One of the interesting studies indicating the prominent role of T cells in the pathogenesis of psoriasis has been done with SCID mice. In the experiment autologous stimulated blood-derived immunocytes from a psoriatic patient were injected under a non-lesional psoriatic skin transplant and the immunocytes were able to induce the conversion of the non-lesional skin to psoriatic plaque. If the transplanted skin originated from a healthy person, a similar effect was not observed (Wrone-Smith&Nickoloff 1996).



### 2.2.3 The Rag2/Rag1 mouse

To obtain a mouse model with SCID-like phenotype without problems with the reversibility, deletion of recombinase-activating genes 1 and 2 (Rag1 and Rag2), participating in the development of the T and B cells, can be used (Boehncke&Schon 2007). If deficiency in the innate immune system is also needed, SCID or Rag-deficient mice can be crossed with mice strains deficient in the innate immune system. This enhances tolerance to the xenografts. Another mice strain used in xenograft studies is AGR129, which is a triple mutant lacking both interferon type I and type II receptors, as well as Rag2, causing T and B cells to be lacking and immature natural killer cells with impaired cytotoxic activity. When psoriatic non-lesional skin was grafted to AGR129 mice, 90 % of the grafts spontaneously developed a psoriasis phenotype with the expansion of T cells within the graft. A similar reaction was not observed if the grafts originated from a healthy person (Boehncke&Schon 2007).

### 2.3 *Genetically engineered mouse models*

A wide variety of genetically engineered mice have been developed to facilitate psoriasis research including both the research of the disease mechanism behind the disease as well as development of new psoriasis treatments. Genetically engineered animals include both transgenic and knockout models and manifest some of the pathological features observed in psoriasis. Enhanced or reduced expression of specific genes is usually directed to the basal epidermis or suprabasal layer with specific keratin gene promoters. Promoters from keratin-5 (KRT5) and keratin-14 (KRT14) direct the expression to the basal epidermis as keratin-1 (KRT1), keratin-10 (KRT10) or involucrin promoters direct the expression to the suprabasal layer (Gudjonsson&Elder 2007). Genetically modified models include a wide spectrum of target genes (Table 1) and can be divided into three categories: modifications targeted to epidermal keratinocytes, leukocytes or vascular endothelium (Danilenko 2008). So far the best psoriasis models among genetically engineered mice are those with targeted mutations in keratinocytes and vascular endothelium. Mice with JunB/c-jun, Stat3, *cdsn* and *Vegf* mutations present many of the typical psoriatic features and most of all they all can develop lesions with psoriatic characteristics spontaneously without outside stimulus. Interestingly, genetic engineering attempts targeted at T cells have not been able to produce a mouse model representing hallmark features of psoriasis, despite the profound role T

lymphocytes have been proposed to have in immunological processes, participating in the pathogenesis of psoriasis. Hence, genetically modified mouse models developed so far emphasize the role of keratinocytes and vascular vessels in the multifactorial pathogenesis of psoriasis.

### 2.3.1 c-Jun/JunB knockout mouse

An example of a mouse model with modifications in epidermal keratinocytes is the c-Jun/JunB knockout mouse. Jun proteins are part of the homo- or heterodimeric transcription factor complex AP-1, which also includes members from Fos, activating transcription factor (ATF) and musculoaponeurotic fibrosarcoma (Maf) protein families (Eferl&Wagner 2003). Jun family members, namely c-Jun (Jun), JunB and JunD, are important regulators of keratinocyte proliferation and differentiation and also have a profound role in cytokine production, all processes relevant for the pathogenesis of psoriasis (Zenz&Wagner 2006). Despite the high structural uniformity between the Jun proteins, they show significant differences in DNA binding activities and in transcriptional activation properties. C-Jun is regarded as a positive regulator of keratinocyte proliferation and differentiation through its direct effect on the expression level of epidermal growth factor receptor (EGFR). JunB antagonizes the effects of c-jun on proliferation. The DNA binding activity of AP-1 protein complexes is decreased in psoriatic plaques (Johansen et al. 2004). Interestingly, JunB gene is located to PSORS6 locus on chromosome 19p13.

Zenz *et al.* have engineered JunB/c-jun deficient transgenic mice to study the effects of AP-1 downregulation (Zenz et al. 2005). To avoid embryonic lethality due to AP-1 deletions (Zenz&Wagner 2006), studies were conducted in an inducible and conditional manner. Both JunB and c-Jun knockouts as well as double knockout including JunB/c-Jun were engineered, but only the double mutant showed spontaneous psoriasis phenotype emerging ten days after activation of the deletion. Mice skin presented the histological hallmarks of psoriasis, including a strongly thickened epidermis with prominent rete ridges, thickened keratinized upper layers with parakeratosis and increased supraepidermal vascularization. Also the immunological picture resembled that observed in psoriasis. In addition arthritic lesions resembling psoriasis arthritis were observed in the double mutant mice. Gene expression profiling revealed that the expression profile of the c-Jun/JunB mice resembled that observed in psoriatic cells. For example expression of chemotactic proteins S100A8 and S100A9 was upregulated, and keratin 15 and caveolin expression was downregulated.

Upregulation of s100a8 and s100a9 was the first phenomenon observed in the pre-diseased skin. JunB/c-Jun deletions were also introduced to Rag-2 deficient mice and the phenotype was only slightly milder in the absence of functional T cells. Also the chemokine profiles of the two mouse types unquestionably promoted the idea that the presence of T cells was not an absolute prerequisite for the development of psoriasis. The JunB/c-Jun knockout mouse is one of the most complete animal models for psoriasis, spontaneously developing a psoriasis-like condition with relevant immunological and keratinocyte effects.

### 2.3.2 Transgenic K5-Stat3 mouse

Another interesting transgenic mouse model for psoriasis is transgenic mouse with constitutively active Stat3 overexpression. Stat3, a member of the signal transducer and activator of transcription aka Stat protein family, participates in cell proliferation, apoptosis, cell differentiation and other important biological functions (Yu&Jove 2004). In addition, its expression is elevated in psoriasis and in lesional keratinocytes, particularly in the nuclei of keratinocytes (Bowcock et al. 2001; Sano et al. 2005a; Sano et al. 2005b). Stat3 is activated by many different cytokines, including members of IL-20 subfamily cytokines, namely IL-19, IL-20, IL-22 and IL-24, which are all upregulated in psoriatic skin (Sa et al. 2007). Of these, IL-22 is produced by Th17 lymphocytes, in contrast to the other related cytokines, and its production is induced by IL-23. Overexpression of IL-23 is profound in lesional psoriatic skin and Stat3 activation via IL-22 seems to be important in the IL-23-induced epidermal acanthosis relevant in the pathogenesis of psoriasis (Zheng et al. 2007).

Sano *et al.* have engineered a Stat3 transgenic mouse, which expresses Stat3 constitutively under keratin 5 promoter, targeting expression to the basal layer of epidermis as well as to keratinocyte stem cells (Sano et al. 2005a). Stat3 mice were phenotypically normal until the age of two weeks. After that their skin became reddened and scaly and development of hyperkeratotic lesions was observed in the tail, dorsum and hind feet. The epidermis of the affected tails showed marked hyperplasia with elongation of rete ridges, confluent parakeratotic scale and loss of the granular layer. In addition, dense dermal infiltrate of inflammatory cells, increased number of capillaries and neutrophils were present in the epidermis. The keratin expression profile with decreased keratin 1 and upregulated keratin 6 expression in suprabasal layer reminisced that observed in human psoriatic skin, suggesting keratinocyte differentiation alterations similar to those found in human psoriasis.

One can conclude that the histological features of Stat3 transgenic mice resembled many of the features found in human psoriatic skin. Plaques were also formed after wounding, suggesting that constitutive activation of Stat3 increased the sensitivity of the epidermis to external stimuli, leading to psoriatic phenotype. This is in concordance with human psoriatic skin, as it may also develop a plaque after physical trauma according to Koebner's phenomenon. Plaque formation after mild wounding was T cell-dependent. When Stat3 expression was inactivated with topical Stat3-specific oligonucleotide decoy treatment, plaques did not develop in response to mild wounding. As Stat3 is a transcription activator, it regulates expression levels of various target genes. In the Stat3 transgenic mouse, expression of many genes, including *Icam1* and *Vegf*, were upregulated. Upregulated *Vegf* expression was consistent with the thicker and more prominent subcutaneous blood vessels in Stat3 mice. Overexpression of *Icam-1* promotes the role of T cells in plaque formation of Stat3 mice, as *Icam-1* has a role in recruiting T cells to inflamed skin (Sano et al. 2005b). Transgenic Stat3 mouse model recapitulates many of the psoriatic features and is one of the most complete psoriasis animal models existing today.

#### 2.3.4 Corneodesmosin knockout mouse

A mouse with a targeted deletion of the corneodesmosin gene (*Cdsn*) is another highly interesting example of genetic engineering targeted at keratinocytes (Matsumoto et al. 2008). *Cdsn* is a psoriasis candidate gene located to the PSORS1 locus (Chang et al. 2006). Corneodesmosomes are the modified desmosomes of the uppermost layer of epidermis participating in corneocyte cohesion and are essential for the proper desquamation of skin. *Cdsn*-deficient mice were born alive and grossly appeared normal, indicating that *Cdsn* is dispensable for embryogenesis. However, *Cdsn*-deficient mice died of dehydration caused by defective skin barrier function a few hours after birth. The mice did not have any other major pathological changes. The upper epidermis was missing due to detachment of the stratum corneum from the underlying granular layer or within the upper granular layer. Also the capillaries beneath the affected epidermis were dilated. In addition the mice had impaired hair growth. To study the long-term effects of *Cdsn* deficiency, skin samples from the *Cdsn* knockout mice were grafted on nude mice. Four weeks after grafting, the samples showed a number of psoriasis-like features, including acanthosis, hyperkeratosis, parakeratosis, loss of granular layer, severe dermal infiltration of inflammatory cells, hyperproliferation, dilation of capillaries and deficient formation of cornified envelope. Also structures resembling rete ridges were seen. Interestingly, Stat3 protein was partially

translocated to the keratinocyte nuclei of Cdsn-deficient skin grafts, suggesting its activation. Activated Stat3 may be relevant in contributing to the psoriatic changes observed in grafted Cdsn-deficient tissue. It is noteworthy that psoriasis-like changes developed spontaneously (Matsumoto et al. 2008). The mouse model with deficient Cdsn gene highlights the fact that Cdsn is relevant for keratinocyte proliferation and differentiation. Its relevance as a psoriasis candidate gene is hindered by the observation that CDSN mutations in humans cause a hair loss or baldness phenotype without any clinical characteristic of psoriasis (Levy-Nissenbaum et al. 2003).

### 2.3.5 Transgenic K14-Vegf mouse

Transgenic vascular endothelial growth factor (Vegf) mouse is a good example of a psoriasis mouse model with modifications aimed to vascular endothelium. Vegf is an important epidermis-derived vessel-specific growth factor. As its expression is upregulated in psoriatic lesions it is regarded as an indicator of active psoriasis arthritis (Detmar et al. 1994; Fink et al. 2007). Upregulation of Vegf expression leads to increased density of tortuous cutaneous blood capillaries with a high level of Vegf receptor expression. Based on what is known about Vegf and its functions and the role of capillaries in psoriasis, it is reasonable to suppose that Vegf is involved in the development of psoriasis.

Vegf transgenic mice were engineered with keratin 14 promoter, directing expression of the transgene to the basal layer of the epidermis (Detmar et al. 1998; Xia et al. 2003; Hvid et al. 2008). Young Vegf mice are healthy and fertile. However, the ear skin of homozygous mice was redder than that of control mice (Xia et al. 2003). At the age of three months, Vegf mice began to develop skin lesions characterized by erythematous and scaly skin. Interestingly, lesions developed at the sites of highest transgenic Vegf expression. Histologically, three-month-old Vegf mice have mild or pre-psoriatic phenotype with moderate acanthosis, focal parakeratosis, mild rete ridge formation and increased dermal tissue thickness and inflammatory cell infiltration. Also Koebner's phenomenon was observed in the mice after wounding. After five months of age, transgenic Vegf mice developed lesions resembling fully developed human psoriasis plaques with hyperkeratosis and parakeratosis and expression of keratin 6 throughout the hyperplastic epidermis. The vessels of Vegf mice in dermal papillae were enlarged and tortuous and analogous to vessels in human psoriatic lesions. They also show a similar gene expression profile to that observed in human psoriatics with prominent E-selectin, Vcam-1 and Icam-1 expression.

The T lymphocyte infiltrate was also similar to that observed in human psoriatics. The specific role of the Vegf transgene in the formation of psoriasis-like plaques is supported by the fact that the psoriasis phenotype resolved when the function of the transgene was blocked. It is still unclear how Vegf causes the psoriasis-like phenotype, but one explanation is the elevated levels of vascular adhesion molecules like Icam-1, which have a central role in promoting extravasation of inflammatory cells to the skin. Hvid *et al.* have further analyzed K14-Vegf mice with topical TPA treatments and have concluded that Th17-driven inflammation is profound in these mice. Th17-driven inflammation is supported by the presence of p40, a part of a functional IL-23, essential for maintenance and survival of Th17 cells. In addition, other cytokines also detected after TPA treatment, including IL-6, IL-22 and IL-17, strongly support a Th17-mediated inflammation. In contrast to human psoriatic plaques, despite the presence of IL-12, Vegf mice did not promote a Th1 response (Hvid et al. 2008). Altogether, transgenic Vegf mouse represents a psoriasis mouse model with striking similarities to human psoriasis and promotes the role of vascular capillaries in the development of psoriasis.

#### 2.3.6 Transgenic K14-interleukin 12 and 23 mice

Inflammatory cells can also be targeted in transgenic mouse models. One example is a transgenic mouse in which the p40 gene, a subunit of IL-12 and IL-23, was overexpressed in basal epidermal keratinocytes under the K14 promoter. The mice developed cutaneous inflammation but it resembled eczema and atopic dermatitis more than psoriasis, as it lacked cutaneous CD8 positive T cell infiltrate, which is considered a hallmark of psoriasis (Kopp et al. 2001; Kopp et al. 2003; Danilenko 2008).

### 3. Genetics of psoriasis

#### 3.1 Inheritance

A familial component in the etiology of psoriasis has already been known for decades. Nevertheless, the precise picture of the genetic components behind the disease still remains unclear, requiring more research until the final word about the matter is said. The first profound study indicating implication of inheritance in the etiology of psoriasis was conducted in the Faroe Islands by Lomholt (Lomholt 1963). He investigated almost 11,000 people, representing one third of the island's population. The study showed that 91 % of the

patients had an affected family member and that the psoriasis incidence was higher among first- and second-degree relatives of psoriatics when compared to the entire population. Afterwards, studies in other populations have confirmed that genetic background affects the pathogenesis of psoriasis (Farber&Nall 1974; Brandrup 1984; Swanbeck et al. 1994). The risk for offspring to develop psoriasis is 20 % if one of the parents suffers from the disease, and if both of the parents are psoriatics, the risk is 75 %. In addition, when one monozygotic twin suffers from psoriasis, the probability for the other twin to develop psoriasis varies between 35 to 63 % (Brandrup et al. 1982; Duffy et al. 1993; Sabat et al. 2007). Monozygotic twins also share a similar clinical disease course whereas in dizygotic twins disease patterns vary. In dizygotic twins the concordance is considerably lower, varying between 12 to 15%. Based on the twin studies, the total heritability is estimated to be 91% (Barker 2001). The fact that concordance between monozygotic twins is less than 100 % shows that in addition to genetic background, environmental factors also affect the onset of psoriasis. Even though genetic predisposition seems to have an important role in the susceptibility to develop psoriasis, the inheritance pattern that psoriasis follows suggests that psoriasis is a complex disease and its inheritance is multifactorial (Henseler 1997; Sabat et al. 2007).

A considerable amount of genetic research has been done through the years in order to identify the genes behind psoriasis. Genetic susceptibility studies usually involve an attempt to identify the allelic variants significantly associated with the increased risk of the disease under study (Valdimarsson 2007). There have been different kinds of genome wide approaches used for the purpose and linkage analysis was the first. It relies on the fact that marker alleles near a disease gene are co-inherited with the disease gene within a family unless a recombination has occurred. Inheritance of a marker allele is monitored in a family that has both affected and unaffected members. As the knowledge of the variation in the human genome has increased, association analysis has become widely used. In association analysis, the frequencies of alleles with single nucleotide polymorphisms (SNPs) between the cases and controls are analyzed. Lately large genome-wide association studies (GWAS) have become more popular. In GWAS, frequencies of hundreds of thousands of SNPs are compared between cases and controls (Duffin&Krueger 2008). Genome-wide analyses of psoriasis susceptibility genes have indicated the presence of numerous different susceptibility loci.

### 3.2 The *PSORS1* locus

The psoriasis susceptibility 1 locus (*PSORS1*) is the major locus for psoriasis. It is located in the MHC region on chromosome 6p21.3 and the association of psoriasis with the specific HLA-C gene form (HLA-Cw6) in the MHC region has been known for more than 25 years (Tiilikainen et al. 1980). *PSORS1* accounts for 30-50% of the genetic predisposition to psoriasis (Trembath et al. 1997) and the association has been consolidated in different populations, all indicating significant linkage (Nair et al. 1997; Burden et al. 1998; Samuelsson et al. 1999; Lee et al. 2000; Veal et al. 2001; International Psoriasis Genetics Consortium 2003). Interestingly, the strength of the genetic linkage and association with *PSORS1* and psoriasis is among the strongest for any complex disease (Kere 2005). The *PSORS1* risk locus increases the possibility to develop psoriasis by 2.5-fold and it shows a dominant-like inheritance pattern (Asumalahti et al. 2002).

The exact genomic localization of the *PSORS1* effector gene is still unclear (Bowcock&Barker 2003). It was considered most likely that the *PSORS1* gene locates in a region of about 200 kb telomeric from HLA-C (Oka et al. 1999; Nair et al. 2000). The area includes eight genes, namely HLA-C, *POU5F1* (*OTF3*), *TCF19*, *CCHCR1* (*HCR*, *Pg8*), *SPR1*, *SEEK1*, *CDSN* and *STG* (Figure 4).

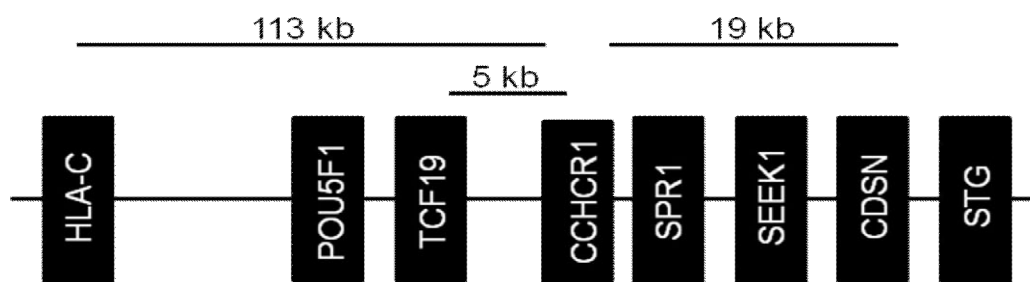


Figure 4. Genes in the *PSORS1* locus on chromosome 6p21.

Genes located in the *PSORS1* region, especially HLA-C, *CCHCR1* and *CDSN* are in strong linkage disequilibrium, which has prominently hindered the genetic studies of *PSORS1*. Even though the original association found was between psoriasis and HLA-C, it has been proposed that HLA-C would be in strong linkage disequilibrium with the actual *PSORS1* gene (Jenisch et al. 1998). Despite the well-characterized association of HLA-Cw6 and



psoriasis and the significant amount of research done to identify the mechanism for the gene to promote pathogenesis of psoriasis, the mechanisms still remain unclear (Bos&De Rie 1999). In addition, the high-risk haplotype bearing HLA-Cw6 may associate with psoriasis more strongly than HLA-Cw6 alone. Neither does the biological role of HLA-C promote it as a PSORS1 effector gene: the HLA-C participates in antigen presentation for natural killer and CD8 positive T cells, whereas the primary pathogenic cell population in psoriatic skin is CD4 positive T cell (Barker 2001). HLA-C is expressed in skin, predominantly suprabasally in the epidermis with a membrane-like expression pattern, and its expression level is elevated in psoriatic plaques (Carlen et al. 2007). In addition to HLA-C, other PSORS1 genes have also been considered as susceptibility genes for psoriasis. According to recent investigations, the most strongly associating SNP in the PSORS1 region was located 34.7 kb upstream from the transcriptional start site of HLA-C (Liu et al. 2008). Thus, despite the shortcomings and lack of a functional understanding for HLA-C in the pathogenesis of psoriasis, many consider it as the strongest PSORS1 candidate gene.

Corneodesmosin (CDSN, S gene) is one of the candidates for PSORS1 gene. CDSN is expressed in terminally differentiated granular keratinocytes and CDSC protein localizes to the modified desmosomes ensuring the intercellular cohesion of keratinocytes (Simon et al. 2001; Jonca et al. 2002). Corneodesmosomes are modified desmosomes in the uppermost layer of the epidermis (Matsumoto et al. 2008). Presence of CDSN is essential for normal desquamation (Chang et al. 2006). CDSN has many psoriasis-associated genotypes including CDNS\*971T, CDNS\*TTC and CDSN\*5, but the evidence supporting CDSN as the effector gene of PSORS1 has shown differences between populations (Asumalahti et al. 2000; Ameen et al. 2005; Martinez-Borra et al. 2005; Chang et al. 2006). Variations between different populations do not support CDSN as the PSORS1 gene.

Corneodesmosin expression is enhanced in psoriatic plaques as compared to healthy or non-lesional psoriatic skin (Allen et al. 2001; Simon et al. 2008). The psoriasis-associated CDSN\*971T form has increased mRNA stability (Capon et al. 2004a). Interestingly, proteolysis of CDSN, a process prerequisite for desquamation, is altered in psoriasis but the defects are independent from the CDSN genotype (Simon et al. 2008). Recently, a mouse model with a targeted deletion in *Cdsn* gene was engineered by Matsumoto *et al.* in order to study the role of CDSN in mouse skin (Matsumoto et al. 2008) (see section “Genetically engineered mouse models). Interestingly, longer-term effects of CDSN deficiency

resembled psoriasis in numerous characteristics. The functional properties of CDSN are highly relevant for psoriasis, but the genetics do not support it solely as the PSORS1 gene. Also, mutations in CDSN have been described in humans in the context of hair loss or baldness, and the patients have no signs whatsoever of a psoriatic phenotype (Levy-Nissenbaum et al. 2003). In addition, according to the latest genome-wide association analysis CDSN does not localize at the region of the strongest PSORS1 association (Zhang et al. 2009).

There are also other genes located in the PSORS1 locus but their importance as the effector gene for PSORS1 appears even less convincing. POU5F1 codes for transcription factor Oct-3/4 that has an essential role in the formation of a pluripotent founder cell population in the mammalian embryo and the amount of Oct-3/4 is highly relevant as different levels induce divergent developmental programs. POU5F1 has been suggested to be a highly important regulator of pluripotency (Niwa et al. 2000). As POU5F1 has a focal role in embryogenesis, including cardiac development (Zeineddine et al. 2006), and its expression levels have profound effects on embryogenesis, it is unlikely that defects in POU5F1 could cause as mild of a phenotype as psoriasis. Transcription factor 19s (TCF19, ac. SC1) expression is regulated according to cell cycle and is classified as a late growth-regulated gene. TCF19 has been proposed to code trans-activating factor, having a role in the transcription of genes required for the late stages of cell cycle progression (Ku et al. 1991), but more evidence is needed to be able to ascertain its exact role. PSORS1C1 and PSORS1C2 (Psoriasis susceptibility 1 candidate gene 1 and 2, also known as SEEK1 and SPR1) are both expressed in healthy and psoriatic skin and have psoriasis-associated SNPs. Association of SPR1 gene with psoriasis is dependent on HLA-CW6. In SEEK1, two SNPs associate with psoriasis independently from HLA-CW6 but the SNPs do not cause an amino acid change and the exon where the SNPs locate is not translated to protein (Holm et al. 2003). Several lines of investigation have suggested that SEEK1 should not qualify as the effector gene of PSORS1 (Rahman et al. 2005; Chang et al. 2006). STG gene is expressed in tonsils and healthy skin but not in keratinocytes or psoriatic skin. STG is a polymorphic gene but the polymorphisms do not associate with psoriasis (Sanchez et al. 2004).

### 3.3 Other psoriasis loci

As PSORS1 accounts for 30-50% of the familial psoriasis cases and its penetrance is 10-15%, existence of other psoriasis susceptibility loci is probable (Bowcock&Krueger 2005). It is likely that there is not only one gene responsible for the pathogenesis of psoriasis, but rather is more likely to be caused by the cooperation of many genes. Presence of at least nine psoriasis susceptibility loci has been proposed based on genome-wide linkage studies and family-based association mapping.

#### 3.3.1 PSORS2

PSORS2 locus is located on chromosome 17q25 and its association to psoriasis has been confirmed in different Caucasian populations (Tomfohrde et al. 1994; Nair et al. 1997; Enlund et al. 1999; Samuelsson et al. 1999). The locus includes four genes, namely SLC9A3R1 (Solute carrier family 9), EBP50, NAT9 (N-acetyltransferase 9) and RAPTOR (Tomfohrde et al. 1994; Speckman et al. 2003; Bowcock&Krueger 2005). Helms *et al.* have shown that PSORS2 has two separate association peaks, one of which is located to the proximity of SLC9A3R1 and NAT9 (Helms et al. 2005). SLC9A3R1 is a phosphoprotein proposed to have a diverse role in the epithelial membrane biology and immune synapse formation in T cells. SLC9A3R1 expression is most prominent in the uppermost spinous cell layer of both psoriatic and normal skin and also in inactive T cells. SNPs found in the close proximity to SLC9A3R1 and NAT9 cause the loss of transcription factor RUNX1 binding. Interestingly, RUNX1 is needed for normal hematopoiesis, including development of thymic T cells, and activates production of IL2 and IFN- $\gamma$  in DC4+ T cells (Ono et al. 2007). A common haplotype carrying SLC9A3R1 and NAT9 has been shown to associate with psoriasis in one US patient population but the risk associated with the haplotype is low, with the odds ratio lower than 1.2. It has been argued that the genetic background behind autoimmune disease includes a combination of relatively common haplotypes or alleles causing the disease (Bowcock&Krueger 2005). PSORS2 also includes another susceptibility locus that encodes RAPTOR gene (regulatory associated protein of MTOR) (Hara et al. 2002; Capon et al. 2004b; Helms et al. 2005). MTOR (mammalian target of rapamycin) is a protein kinase that regulates cell growth and proliferation in response to environmental stimuli and its activation is proposed to be regulated by RAPTOR (Kim et al. 2002). RAPTOR is expressed in a variety of tissues including lesional and non-lesional

psoriatic skin. Variations of RAPTOR predisposing to psoriasis locate to the non-coding region, suggesting a possible regulatory role. However, the relevance of RAPTOR as a psoriasis candidate gene has been questioned (Stuart et al. 2006).

### 3.3.2 PSORS3

Matthews *et al.* found linkage association for psoriasis to chromosome 4q in English and Irish families (Matthews et al. 1996). The PSORS3 locus contains a candidate gene called interferon regulatory factor 2 (IRF2), whose function is relevant for the pathogenesis of psoriasis (Foerster et al. 2004). However the association has not shown replication in other populations (Nair et al. 1997; Enlund et al. 1999; Zhang et al. 2002).

### 3.3.3 PSORS4

PSORS4 has been located to chromosome 1q21 in numerous populations (Bhalerao&Bowcock 1998; Capon et al. 1999; Chen et al. 2009). Interestingly, PSORS4 includes several genes participating in epidermal differentiation as it is located in the epidermal differentiation complex (EDC). EDC is a group of 70 genes expressed during terminal keratinocyte differentiation, including genes encoding S100 proteins, small proline-rich proteins (SPRRs) and late envelope proteins (Bowcock&Krueger 2005). Numbers of genes have been suggested as PSORS4 candidate genes, including S100A8, S100A9 and loricrin which all show allele-specific differences in expression profiles (Semprini et al. 2002; Giardina et al. 2004). Also peptidoglycan recognition proteins (PGLYRP) and involucrin genes have been suggested to associate with psoriasis (Chen et al. 2009; Kainu et al. 2009). Recently one PSORS4 gene, LCE3C, a member of the late cornified envelope gene cluster, was shown to have variability in copy number in psoriatics as they were shown to have a higher percent of reduced number of the gene than healthy individuals (de Cid et al. 2009).

### 3.3.4 Other psoriasis loci

The linkage association of psoriasis to PSORS5 locus was originally detected in a Swedish population (Enlund et al. 1999). PSORS5 is located on chromosome 3q21 and includes the candidate gene SLC12A8, a member of solute carrier family 12 (Hewett et al. 2002; Huffmeier et al. 2005). There are also other interesting genes located in PSORS5. One example is Cystatin A, a gene coding for skin barrier cysteine protein inhibitor whose

expression is upregulated in psoriatic plaques. However, its association to psoriasis has been controversial and the association is dependent on PSORS1 genotype (Samuelsson et al. 2004; Vasilopoulos et al. 2008).

Genome-wide linkage scan with German families indicated the presence of PSORS6 locus on chromosome 19p13 (Lee et al. 2000). The JunB gene is located in PSORS6. JunB expression is downregulated in psoriatic lesional keratinocytes and it has been shown to be relevant for the pathogenesis of psoriasis as the mouse model deficient in JunB/c-Jun develops a psoriasis-like phenotype (Zenz et al. 2005) (see section “Genetically engineered mouse models”).

Another psoriasis susceptibility locus, PSORS7 locus is located on chromosome 1p. The EPS15 gene producing intracellular substrate of the EGF receptor and overexpressed in psoriatic epidermis is located in the area (Veal et al. 2001). Otherwise the locus remains poorly characterized.

PSORS8 locus in chromosome 16q has also been identified (Nair et al. 1997; Karason et al. 2003). Interestingly, Crohn’s disease variant gene NOD2 is also located in 16q. However, studies have eliminated the possible role of NOD2s as a psoriasis candidate gene and more data is needed to clarify the role of PSORS8 in the genetics of psoriasis (Young et al. 2003).

The PSORS9 locus on chromosome 4q31-4q32 was identified in a Chinese Han population (Yan et al. 2007; Zhang et al. 2007). The IL-15 gene is located in the area and it has been pointed out as a susceptibility gene, as a SNP in 3’ UTR of the gene associates strongly to psoriasis and also has effects on the expression pattern of the gene (Zhang et al. 2007).

In addition to the PSORS1-9 loci, other loci have also been suggested to exist. A recent addition to the list of loci associated with psoriasis is a novel psoriasis locus on 2p22.3 - 2p11.2, identified in the Chinese Han population (Sun et al. 2008). Genome-wide association analysis has proposed a novel psoriasis locus to chromosome 4q27, an area harboring genes for interleukin 2 and interleukin 21 relevant in the pathogenesis of many autoimmune diseases (Liu et al. 2008). The variety of psoriasis loci indicates the

multiplicity of genes affecting the disease and also suggests that there may be true differences between different populations.

#### 4. The psoriasis susceptibility gene CCHCR1

The Coiled-Coil  $\alpha$ -Helical Rod Protein 1 (CCHCR1, former HCR and Pg8) was originally predicted from genomic sequence by Guillaudoux *et al.* (Guillaudoux et al. 1998), after which the gene finding was verified and the gene renamed by Oka (Oka et al. 1999). Asumalahti *et al.* further characterized it as a novel psoriasis susceptibility candidate gene (Asumalahti et al. 2000). CCHCR1 is located in PSORS1 region between HLA-C and corneodesmosin (Figure 4) and is in strong linkage disequilibrium with HLA-C both in psoriatics and healthy persons. CCHCR1 gene is highly polymorphic with at least 12 coding variants in addition to non-coding SNPs, both in exon and intron areas, and the gene codes a protein of 782 amino acids. The secondary structure is predicted to include  $\alpha$ -helical coils and its amino acid sequence shows only little homology with other known proteins. Predictions suggest CCHCR1 to be a nuclear protein with a leucine zipper motif (Asumalahti et al. 2000). Four of the coding SNPs form the psoriasis susceptibility allele CCHCR1\*WWCC (SNPs correspond to nucleotides 307, 325, 1723 and 2327), where two arginines have become tryptophans and two glycines cysteines (Figure 5).

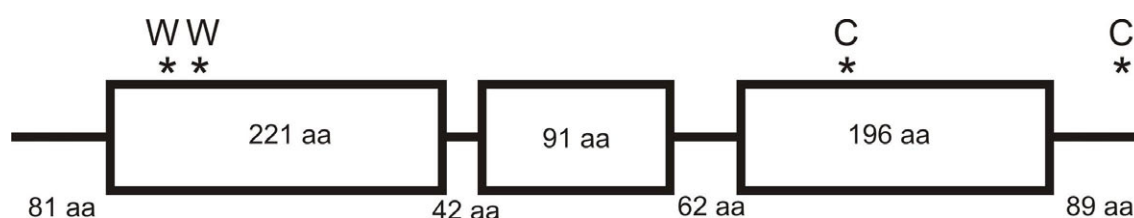


Figure 5. Schematic structure of CCHCR1 protein. Rectangles represent localizations of predicted helical structures, which are flanked by intervening non-coiled structures. Approximate localization of CCHCR1\*WWCC SNPs and the numbers of amino acids (aa) of different domains are presented.

The CCHCR1\*WWCC psoriasis-associated gene form with an odds ratio of 2.5 (1.9-3.3) is found on 35 % of psoriatic chromosomes and 18 % of control chromosomes, suggesting that CCHCR1\*WWCC has a role in the pathogenesis of psoriasis. Relevance of CCHCR1 as a PSORS1 candidate gene has been argued (Chia et al. 2001; O'Brien et al. 2001), but its association with psoriasis has been successfully replicated in Jewish, Chinese and Taiwan Chinese psoriatics, suggesting its relevance in different populations (Chang et al. 2004;

Chang et al. 2005; Martinez-Borra et al. 2005). CCHCR1\*WWCC variant may have an altered secondary structure as the first two amino acids of the genotype predicted affect the secondary structure by decreasing the probability of helical coiled-coil structure (Asumalahti et al. 2002).

CCHCR1 is expressed by a variety of tissues, with the most abundant expression found in heart, liver, skeletal muscle, kidney, testis and pancreas and weaker expression in lung, spleen, thymus, prostate, ovary, small intestine, colon, peripheral blood leukocytes, brain and placenta (Asumalahti et al. 2000; Sugawara et al. 2003). In healthy and non-lesional psoriatic skin, CCHCR1 protein expression is confined to basal keratinocytes and the expression pattern is uniform. In psoriatic lesions, CCHCR1 expression is more abundant and the expression is enhanced within nuclei and cytoplasm of basal keratinocytes and also suprabasally above dermal papillae. Rete ridges are mostly negative for CCHCR1 expression. Interestingly, CCHCR1 expression shows an inverse expression pattern compared to the proliferation marker Ki-67, suggesting a less prominent CCHCR1 expression at the sites of active proliferation (Asumalahti et al. 2000; Asumalahti et al. 2002; Suomela et al. 2003). CCHCR1 is also expressed in some epidermally originating cancers, and interestingly, it shows an inverse expression pattern compared to the hyperproliferation marker Ki-67 also in cancers (Suomela et al. 2003). In cultured lesional keratinocytes, CCHCR1 expression is enhanced and nuclear localization has also been observed. Gene regulation of CCHCR1 has been studied by treating keratinocytes with different psoriasis-related cytokines. However, only INF- $\gamma$  affects CCHCR1 expression by downregulating it (Suomela et al. 2003).

In addition to humans, CCHCR1 gene is also found in other animal species, including *Pan troglody* (common chimpanzee), *Pongo abelii* (Sumatran orangutan), *Mus musculus* (house mouse), *Bos taurus* (European cattle) and *Xenopus tropicalis* (diploid frog). The coding sequence homologies of CCHCR1 between different species vary between 70% and 87 %. The highest homology, 99%, is between chimpanzee and human. *Xenopus tropicalis* is an exception as it does not show any significant similarity to human CCHCR1. The homology of the human and mouse CCHCR1 coding regions as well as amino acid sequence is 72% and interestingly the three first SNPs of CCHCR1\*WWCC allele are located to the homologous areas. In the genomic sequence, the homology between human and mouse CCHCR1 gene is 39 %.

The functional aspects of CCHCR1 have remained poorly understood. CCHCR1 is not expressed by the areas of active proliferation, which suggests a role in keratinocyte proliferation. The dual localization in cytoplasm and nucleus suggests a possibility for a functional role in both cell compartments. In addition, CCHCR1 participates in steroidogenesis as Sugawara *et al.* have shown that CCHCR1 enhance steroidogenesis by interacting with the steroidogenic acute regulatory protein (StAR) (Sugawara et al. 2003). StAR protein plays a key role in the transportation of cholesterol from the outer mitochondrial membrane to the inner membrane, where the first step of steroid hormone synthesis occurs as the cytochrome P450 side chain cleavage enzyme (P450<sub>scc</sub>) facilitates conversion of cholesterol to pregnenolone. StAR expression is confined to steroid-producing tissues, but whether it is expressed in skin was unknown, although steroidogenesis also occurs in skin (Thiboutot et al. 2003; Zouboulis&Degitz 2004; Stocco et al. 2005). Steroid hormones have profound effects in skin, as they are needed for cell proliferation, epidermal barrier homeostasis, differentiation and modulation of immune response, all processes relevant for the pathogenesis of psoriasis (Zouboulis&Degitz 2004; Bikle et al. 2005). Even though something about the function of CCHCR1 is already known, more research is needed to find out its detailed function and relevance for the pathogenesis of psoriasis.



## **AIMS OF THE STUDY**

The overall goal of the study was to investigate the function of CCHCR1 and its relevance for the pathobiology of psoriasis by mouse models and functional studies with cultured cells. The specific aims of the study were:

- I        To establish and characterize transgenic CCHCR1 mice expressing either the non-risk or risk form of CCHCR1 and to study the role of CCHCR1 in processes relevant in the pathogenesis of psoriasis, such as keratinocyte proliferation.
  
- II       To investigate the role of CCHCR1 in the steroidogenesis of skin and its interaction with the StAR protein.
  
- III      To study CCHCR1 expression and its regulation in cultured keratinocytes in the context of psoriasis.

## MATERIAL AND METHODS

### 1. Experiments with transgenic CCHCR1 mice

#### 1.1 Transgenic constructs and production of transgenic mice (I)

Two types of constructs were engineered in order to produce transgenic CCHCR1 mice, producing either the normal human allele of CCHCR1 or the psoriasis-associated CCHCR1\*WWCC risk allele under the control of the K14 promoter. Cloning of the normal human CCHCR1 allele was done by Asumalahti *et al.* (Asumalahti et al. 2000; Asumalahti et al. 2002). Risk allele cDNA was obtained by reverse transcriptase PCR (RT-PCR) using RNA isolated from a skin biopsy of a psoriasis patient with a CCHCR1\*WWCC risk allele. The primers used in RT-PCR were the following: forward primer containing a BamHI site 5'-CGCGGATCCCTTTCAACTCTGCCAAGA-3' and reverse primer 5'-CCAC CCACTTCTCCAGGAT-3'. Normal and risk form cDNAs of CCHCR1 were first cloned into the pCMV5 vector, from which they were transferred to the K14 expression vector PG3Z-K14 cassette (Gat et al. 1998). Before transfer the SphI and HindIII restriction sites at 3' side of the polyA in the pG3ZK14 were used to introduce additional SmaI and SacI sites, in order to allow the recovery of linear K14-CCHCR1 fragment for pronuclear injection. For that purpose, a restriction cassette of 37 bp containing sites for SphI, SmaI, SacI and HindIII was synthesized using the oligonucleotides 5'-GCAGCATGCTGCCCCGGGGAGAGCTCCTCAAGCTTCTT-3' and 3'-CGTCGTAC GACGGGCCCCCTCTCGAGGAGTTCTGAAGAA-5'.

Using BamHI sites, the CCHCR1 cDNAs, including the entire coding regions flanked by 52 bp 5' and 42 bp 3' untranslated sequences, were transferred from pCMV5 vector into pG3ZK14. Restriction mapping and direct sequencing were used to validate the orientation of CCHCR1 inserts and fidelity of the sequence. The linearized transgenic fragments containing the K14 promoter, b-globin intron, human CCHCR1 cDNAs and polyA were released from the vector with SacI. Fragments were purified with agarose gel electrophoresis combined with Gel Extraction Kit (Qiagen). The fragments were eluted with ultrapure buffer containing 10 mM Tris (pH 7.5) and 0.2 mM EDTA. The transgenes were microinjected into the pronuclei of fertilized oocytes of FVB/N mice (Jackson Laboratories, Bar Harbor, USA). Altogether, 81 mice with non-risk allele constructs and 75 with risk allele constructs were obtained for further screening. K14-CCHCR1 founder mice

were crossed with the FVB/N mice in order to establish a co-isogenic transgenic line. Tissue specimens for RNA, protein and histological analyses were collected at different ages (1, 2, 4, 6 and 12 months). All animal studies were approved by the regional committee for animal experiments at the University of Helsinki (license number STU 27 A/2002).

### *1.2 Screening of transgenic mice (I)*

Potential founders and littermates were screened for transgene integration by PCR. Genomic DNAs were isolated from tails, digested in 40 mM Tris-HCl, pH 8.0, 20 mM EDTA, 200 mM NaCl, 0.5 % SDS, 0.5 %  $\beta$ -mercaptoethanol and 20 mg/ml proteinase K at 60 °C overnight. After digestion, the DNAs were precipitated with isopropanol, washed with ethanol and dissolved in water. Samples were analyzed by PCR using a forward primer annealing to K14 sequence (5'-ACATCCTGGTCATCATCCTGCC-3') and a reverse primer annealing to CCHCR1 sequence (5'-CTAGCCGCCTCTCTGAGACATC - 3'). The PCR results of founder animals were verified with Southern blotting by using a K14 promoter specific fragment of 795 bp as a probe. The K14-fragment was generated by PCR using a forward primer 5'-AAGCCTGGGCAATAACAATG-3' and a reverse primer 5'-GAAAGCCCCAAAACACTCCAA-3'. Southern blotting was performed according to standard protocols and blots were visualized by autoradiography.

### *1.3 Wounding experiments (III)*

Age matched adult mice homozygous for the risk or non-risk CCHCR1 alleles were used for the experiments. Heterozygote mice from non-risk lines 12 and 34 or risk lines 106 and 132 were crossed to produce homozygote mice used in the experiments. Mice were anesthetized with ketamine hydrochloride (50 mg/kg sc) and xylazine hydrochloride (10 mg/kg sc). Two to three circular full-thickness wounds 5 mm in diameter were made using a punch biopsy device on both sides of the previously shaved backs of the anesthetized mice. Mice were sacrificed after 1, 4, 11, and 30 days post wounding and wound areas were measured. At each time point, four animals from each mouse group were used for analyses. After harvesting, tissues were fixed in neutral formalin, dehydrated and embedded in paraffin. Hematoxylin and eosin (H&E)-stained sections were photographed and analyzed with Olympus BX41 microscope and computer using the Olympus DP-soft version 3.2 image program. Eight wounds per genotype were used for the analyses. Re-

epithelialization was estimated on skin sections by measuring the distance between the original wound site (the edge of the muscular layer) and the leading edge of the epithelium.

#### *1.4 TPA treatment (III)*

Shaved backs of the age matched, three-month-old mice were treated with 10 µg 12-O-tetradecanoylphorbol-13acetate (TPA, Sigma) in 100 µl acetone. The solution was applied over an area of approximately 2x2 cm. At each time point, four animals from each group were used and the animals were sacrificed at defined time points (24, 48 or 72 h) after which the skin samples were processed for histological analysis. Two skin cross-section samples from each mouse were analyzed as the epidermal thickness from the bottom of the stratum corneum to the basement membrane of the interfollicular epidermis was measured. Four measurements on four to seven fields per mouse were made using Olympus BX41 microscope and computer using the Olympus DP-software version 3.2 image program.

#### *1.5 In vivo cell proliferation assay with untreated mice (III)*

Proliferation of epithelial cells was measured by intraperitoneal injection of BrdU (Roche) 50 mg/kg body weight. Four mice were used for each time point in each group and mice were sacrificed 2 or 6 hours after injection. BrdU incorporation was detected by immunohistochemical staining with mouse anti-BrdU monoclonal antibody (Dako). The number of BrdU positive cells in relation to the total number of epidermal keratinocytes was determined from two to three sections per animal and on five to seven fields of each section.

## **2. Experiments with transgenic primary mouse keratinocytes**

### *2.1 Isolation and culture of primary mouse keratinocytes (II, III)*

Keratinocytes from 0- to 3-day-old transgenic CCHCR1 mice (I) or from wild-type animals were isolated. Mice were sacrificed and the skin was removed and incubated in 0.25 % dispase in EBSS overnight at 4 °C. On the following day the epidermis was separated from the dermis, washed, and incubated in 0.25% trypsin solution for 15 min in 37 °C. Epidermal pieces were suspended by pipetting in keratinocyte growth medium

(KGM-2 media supplemented with growth factors, Clonetics, Cambrex Bio Science Walkersville, Inc., MD, USA) supplemented with 8 % decalcified FCS and penicillin-streptomycin. After suspension, the cells were centrifuged (200 x g, 10 min). The pelleted keratinocytes were resuspended in KGM-2 medium supplemented with 2 % decalcified FCS and penicillin-streptomycin and seeded in plates for further cultivation. On the following day the media were changed to KGM-2 without serum. All animal studies were approved by the regional committee for animal experiments at the University of Helsinki (license number STU402A).

### *2.2 Proliferation assay of primary mouse keratinocytes (III)*

For the cell proliferation assays, isolated primary mouse keratinocytes (see previous section) were resuspended in KGM-2 medium supplemented with 2% decalcified FCS and penicillin-streptomycin, counted and 5,000 or 10,000 cells were seeded in 96-well plates. Cells were cultured in the presence or absence of EGF (15 ng/ml, Sigma) for 2-3 days, after which cells were labeled for 2, 4, 8 or 24 h with 10  $\mu$ M BrdU (Roche). Colorimetric ELISA System (Roche Diagnostics, Germany) was used to determine the amount of incorporated BrdU and the assays were done according to the manufacturer's instructions. Absorbance (450 nm) values were plotted as a function of time, and proliferation rates ( $\Delta A_{450 \text{ nm}}/\Delta h$ ) were estimated from the slope of the line.

### *2.3 In vitro cell migration assay (III)*

For the cell migration assays, keratinocytes from newborn mice were isolated (see section "Isolation and culture of primary mouse keratinocytes"), counted and plated in 6-well plates or in Transwell inserts (8  $\mu$ m membrane, Costar) coated with 10  $\mu$ g/cm<sup>2</sup> rat collagen type I (Sigma). Cells in 6-well plates were cultured in the presence or absence of 15 ng/ml EGF (Sigma) 2-3 days and used for the experiments when they were 70-90% confluent. To examine cell motility, a cell-free area was introduced by scraping the cell layer with a pipette tip and after 24 and 48 h of migration, the cell-free area was evaluated (Matthay et al. 1993; Cha et al. 1996). Photographs were taken with a phase contrast microscope. Cells in Transwell chambers were let to migrate 24 or 48 h after which migrated cells were fixed with methanol. Hematoxylin-stained inserts were mounted and migrated cells were counted by microscope. The rate of re-epithelialization was studied

from mouse skin explants with fluorescence imaging technique (Lu&Rollman 2004; Mirastschijski et al. 2004).

### **3. Expression studies of CCHCR1**

#### *3.1 Cell cultures for regulation studies (II)*

HaCaT keratinocytes were cultured and treated with different hormones and chemicals to study regulation of CCHCR1. Cells were seeded on 6-well plates and cultured in DMEM (Gibco, High glucose) with 10 % FCS and before experiments cells were cultured without serum overnight. Subconfluent or confluent cells were treated with estradiol ( $10^{-9}$  –  $10^{-6}$  M, Sigma), insulin ( $10^{-7}$  -  $10^{-5}$  M, Sigma), forskolin ( $10^{-6}$  - $10^{-4}$  M, Sigma), cyclosporine (1-100 ug/ml, Sigma), progesterone ( $10^{-9}$ - $10^{-5}$  M, Sigma), 5 $\alpha$ -dihydrotestosterone ( $10^{-8}$  –  $10^{-6}$  M Sigma), Cyclosporin A (5-100  $\mu$ g/ml, Sigma) and 3,3',5-triiodo-L-thyronine sodium salt (Sigma  $10^{-9}$  -  $10^{-6}$ M) for 24 or 48 hours. Experiments included triplicate samples from each treatment group and experiments were repeated three times. Mock treated cells were used as controls. Also transient cholesterol depletion was carried out by incubating serum-starved cells with 7.5 mM methyl- $\beta$ -cyclodextrin (M $\beta$ CD) (Sigma) for 1 h followed by 17 h incubation in serum-free culture medium. Chronic depletion of cellular cholesterol was done with 1 hour M $\beta$ CD treatment, followed by inhibition of cholesterol biosynthesis with 10  $\mu$ M lovastatin (Sigma) treatment for 17 hours (Jans et al. 2004). In addition cultured HaCat cells were also treated with EGF (10 ng per ml) for 24h prior to use in western analyses.

#### *3.2 mRNA expression analyses*

##### *3.2.1 Isolation of RNA for RT and quantitative real time PCR (I, II)*

Total RNAs were isolated from cultured keratinocytes or skin samples with RNAeasy Total RNA Isolation Kit (Qiagen, Chatsworth, California) according to manufacturer's protocol including DNase I treatment. RNA was reverse-transcribed to cDNA with TaqMan Reverse Transcription Reagents (Applied Biosystems) and used as a template for RT-PCR.

### 3.2.2 RT-PCR (II)

StAR expression was studied with RT-PCR from HaCat cells and human primary keratinocytes. In the PCR assays, StAR specific primers (5'-CCTACAGACACATGCGCAAC-3' and 5'-GCTCCACGAGCTCTTCATAGA-3') were used with 25 cycles and with an annealing temperature of 55 °C. The PCR product was used as a template for StAR re-amplification and the re-amplification product was analyzed on agarose gel. GAPDH was used as a housekeeping control gene and was amplified with the primers 5'-ACGGATTTGGTCGTATTGGG-3' and 5'-TGATTTTGGAGGGATCTCGC-3'.

### 3.2.3 Quantitative real-time PCR (I, II)

Real time quantitative PCR assays (TaqMan) were done with the ABI PRISM 7700 Sequence Detector System (Applied Biosystems). Reaction conditions were programmed on a Power Macintosh 7200 directly linked to the sequence detector. PCR amplifications were performed according to manufacturer's recommendations. CCHCR1 gene expression was analyzed with human system (Hs00219401\_m1, Applied Biosystems) and the vitamin D receptor gene was analyzed with human or mouse systems (Hs00172113\_m1, Mm00437297\_m1, Applied Biosystems). In the quantitative real time PCRs done to confirm microarray results, the expression of the following murine genes was studied: keratin 6a, Mmp9 and S100A10 with pre-designed primer and probe sets (Assay-on-Demand gene expression product, Applied Biosystems) according to the manufacturer's protocols. GAPDH and ribosomal RNA were used as endogenous control genes in quantification (Human GAPDH 20X and Human 18S rRNA 20X and Rodent GAPDH Control Reagents, Applied Biosystems).

## 3.3 *Protein expression analysis*

### 3.3.1 Immunohistochemical analysis (I, II, III)

Murine epidermal samples for immunohistochemistry were taken from various locations. Tissue samples were fixed in 10% formalin solution, embedded in paraffin and sectioned. For histological analysis the sections were stained with hematoxylin and eosin. For immunodetection of human CCHCR1 protein in transgenic mouse skin tissue, the sections were pre-treated with trypsin (10mg/ml) and the localization of CCHCR1 was detected with

affinity-purified rabbit anti-CCHCR1 IgG (7 µg/ml) (Asumalahti et al. 2002). As a control for CCHCR1, stainings with rabbit pre-immune sera (10 mg/ml) or normal rabbit immunoglobulin were performed. The following antibodies were used in immunostaining: polyclonal antibodies against the proliferation marker Ki67 (Novocastra), mouse cytokeratin 5 (Berkeley Antibody Company), filaggrin (Berkeley Antibody Company) and T-lymphocyte marker CD3 (Novocastra, United Kingdom), mouse monoclonals for cytokeratin 6 (Labvision co, Neomarkers), bromodeoxyuridine (Dako) and  $\alpha$ -smooth muscle actin (Sigma), and rabbit polyclonals for cytokeratin 10 (Covance, PRB159P), Ki67 (Novocastra, NCL-Ki67p), laminin 5 (Pyke et al. 1994), von Willebrand factor (DakoCytomation) and CCHCR1 (Asumalahti et al. 2002). For the Ki67 and BrDU stainings, samples were pre-treated by microwaving in citrate buffer. Immunostainings were performed with the avidin-biotin-peroxidase complex technique (Vectastain ABC Kit for CCHCR1; Vector laboratories, Inc., Burlingame, CA and StreptABComplex/HRP Duet (Mouse/Rabbit) Kit for Ki67; DAKO, A/S Glostrup, Denmark, no. K0492). Aminoethylcarbazole or diaminobenzidine were used as a chromogenic substrate. The tissues were counterstained with hematoxylin. Cell apoptosis was visualized using the TUNEL labeling system ApoTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon).

Affinity-purified rabbit immunoglobulins anti-CCHCR1 IgG (4 µg/ml: Asumalahti et al. 2002) and anti-StAR (ABR, Affinity Bioreagents PA1-560, 1:200, USA) were used according to a standard procedure to detect CCHCR1 and StAR from frozen or paraffin human samples including sections of normal skin ( $n = 3$ ), non-lesional and lesional psoriatic skin ( $n = 6$ ), testes ( $n = 4$ ), kidney ( $n = 5$ ), placenta ( $n = 4$ ), and adrenal gland ( $n = 3$ ).

### 3.3.2 Staining of transfected cells (II)

COS-1, A431 and HeLa cells were transiently transfected with the pcDNA3.1/V5-His-StAR, pCMV5-CCHCR1 (non-risk and risk form), pGFP-CCHCR1 (non-risk form) and pHcRedMLN64 constructs using FuGENE 6 reagent (Roche Diagnostics, Germany) according to the manufacturer's instructions. Cells were fixed with 4% paraformaldehyde-PBS solution followed by a PBS wash and permeabilization with 0.1% Triton-X100. Cells were immunostained with antibodies against CCHCR1 (affinity-purified rabbit immunoglobulins 4 µg/ml) (Asumalahti et al. 2002), endosomal markers EEA1 (anti-early



endosomal autoantigen 1, BD Biosciences, San Jose, CA) and transferrin receptor (Zymed) and against the lysosomal marker Lamp1 (Anti-lysosome-associated membrane protein from Developmental Studies Hybridoma Bank, University of Iowa). The mouse monoclonal antibody against V5-tag (Invitrogen) was used to detect the StAR protein. Fluorescently conjugated secondary antibodies (Sigma, Steinheim, Germany) were used as secondary antibodies according to the manufacturers' instructions. Fluorescent Nile Red (0.1-0.5  $\mu\text{g/ml}$  Sigma) was used to stain neutral lipid droplets. Filipin staining and dextran endocytosis were performed as described before (Hölttä-Vuori et al. 2005). To visualize the free cholesterol distribution, cells were fixed and incubated with the fluorescent sterol-binding antibiotic filipin (0.05% in PBS) for 15 min followed by PBS washing. Fluorescent NBD-cholesterol (Molecular Probes, N1148, 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3 $\beta$ -ol) was used for detection of lipid droplets: transfected cells were labeled from 30 min to 18 h with medium containing 1-5  $\mu\text{g/ml}$  NBD-cholesterol at 37 °C in a CO<sub>2</sub> incubator. After a PBS wash, cells were fixed with 4 % paraformaldehyde prior to use for CCHCR1 immunostaining.

### 3.3.3 Western blot analysis (I, II)

Western blot analyses were done to verify CCHCR1 expression in the skin of transgenic CCHCR1 mice. After biopsy, mouse skin was frozen in liquid nitrogen and stored at -70 °C. Tissues were ground with a mortar and pestle in the presence of liquid nitrogen. Tissue powders were homogenized into Laemmli buffer with a syringe with needle. Samples were boiled for 3 min and centrifuged for 10 min at 4°C. To detect CCHCR1 expression from HaCat cells, cells were homogenized into Laemmli buffer with a syringe with needle. The total protein amounts were analyzed with Bradford's method and they varied between the experiments in the total skin samples, from 10 to 40 mg, and in the cell lysates, from 20 to 30  $\mu\text{g}$ . Protein samples were analyzed by SDS-PAGE in the presence of  $\beta$ -mercaptoethanol. Western blot analysis was carried out by using 7.5 % SDS-polyacryamide gels according to manufacturer's recommendation. A rabbit anti-HCR serum (Asumalahti et al. 2002) was used to detect CCHCR1 and the signals were detected with chemiluminescence (ECL, Amersham Pharmacia).

## **4. Other functional CCHCR1 studies**

### *4.1 Microarray expression profiling*

#### 4.1.1 Preparation of cRNA for microarray analysis (I)

Total RNA samples from dorsal skin samples of different mouse groups were used for microarray hybridization. Two-month-old animals from lines 12, 34, 106 and 132, altogether 3 wild-type, 4 non-risk and 4 risk allele animals, were used for the analysis. Mouse skin was washed with 70% ethanol and an approximately 2 cm<sup>2</sup> piece of the back skin was cut for RNA isolation. The samples were rinsed with 70% ethanol and sterile PBS and frozen with liquid nitrogen. Total RNAs were isolated using TRIzol (Invitrogen) according to the manufacturer's recommendations. Isolated RNAs were further purified with the RNeasy total RNA isolation kit (Miniprep-Kit Qiagen, Chatsworth, CA) according to the manufacturer's instructions. To avoid genomic DNA contamination, the RNA samples were treated with DNase I (Qiagen). The quality of RNA was checked by spectrophotometry (A260/280 ratio), with agarose gel electrophoresis and the Agilent Bioanalyzer (Agilent Technologies Inc.). Double-stranded cDNA was synthesized from total RNA and the cDNA was used as a template in *in vitro* transcription to generate biotinylated cRNA. Eight µg of biotinylated cRNAs were hybridized to each array and scanning was performed after biotin/avidin/phycoerythrin amplification.

#### 4.1.2 Array analysis (I)

U74Av2 murine genome Genechip probe array (Affymetrix Inc, Santa Clara), containing probe sets representing approximately 12,000 genes (6,000 well-characterized genes and 6,000 expressed sequence tags, ESTs), was used. The labeled cRNA was fragmented and hybridized to probe arrays according to Affymetrix Expression Analysis technical manual, P/N 700218 rev.2. The probe arrays were washed and stained. GeneChip 3.2 software (Affymetrix Inc.) was used for image scanning, converting obtained intensities to a numerical format and gaining an average difference value for each probe in the array. Experimental procedures were done by following the standard protocols provided by Affymetrix Inc (Santa Clara, CA).

#### 4.1.3 Data analyses (I)

For construction of gene expression profiles, scanned output files were analyzed with the Affymetrix Microarray Suite 5.0 (Affymetrix Inc.). Single array analysis and comparison analysis were used to build databases of gene expression profiles and estimates of gene expression changes. Each chip was normalized for all probe sets to a target intensity of 100 in order to minimize discrepancies between an experiment and baseline array due to experimental variations to allow an intermarry comparisons. Comparisons were performed between 8 transgenic mice and 3 wild-type mice, as well as between the two types of transgenic lines. 24 and 16 comparisons were formed from each of the above comparisons for further analysis. Differentially expressed genes were defined based on t-test results ( $p \leq 0.001$ ) and fold changes were determined using average values of signal log ratios (absolute value  $\geq 1$ ). Genes were excluded if their expression, as defined by the Affymetrix software, was absent in all 11 arrays or if there was no change detected in all comparisons.

To classify genes and to see how well those genes can characterize different mouse lines, cluster analysis was performed using GeneSpring 5.1 software (Silicon Genetics, Redwood City, CA, USA). Two-dimension hierarchical trees were built based on signal intensity values from single array analysis of Microarray Suite after normalization. Similarity of cluster was measured by standard correlation. Subgroups of genes were used for cluster analysis to focus on specific biopathways. Related biological pathways were selected from BioCarta (<http://www.biocarta.com/index.asp>) and altered biological processes were determined by changed gene expression profiles by t-test ( $p \leq 0.001$ ). Information on human orthologs was obtained via Affymetrix annotation tools ([http://www.affymetrix.com/analysis/download\\_center.affx](http://www.affymetrix.com/analysis/download_center.affx), March 2003).

#### 4.2 Steroidogenic pregnenolone assays

##### 4.2.1 Constructs (II)

Cloning of human *CCHCR1* cDNAs corresponding to normal allele (Asumalahti et al. 2000; Asumalahti et al. 2002) and psoriasis-associated risk allele *CCHCR1\*WWCC* (See section “Transgenic constructs and production of transgenic mice”) have been described elsewhere. Both cDNAs were cloned into the pCMV5 vector. *StAR* cDNA was generated by PCR using human testis cDNA (Clontech) as a template. 5’-

ACAATGCTGCTAGCGACATTCAAG-3' primer was used as the forward primer and 5'-ACACCTGGCTTCAGAGGCAGG-3' either with or without the stop codon as the reverse primer. *StAR* cDNA was cloned into pcDNA3.1/V5-His vector (Invitrogen). The F2 construct encoding genes for cytochrome P450scc system was kindly provided by Dr. Walter L. Miller (University of California, San Francisco). Wild-type *MLN64* was described previously (Alpy et al. 2001) and fluorescent fusion construct of *MLN64* was cloned in pHc-Red1-N1 vector (Clontech).

#### 4.2.2 Pregnenolone assays (II)

COS-1 cells cultured on 6-well plates were transiently transfected with the F2 construct, pcDNA3.1/V5-His-*StAR*, pCMV5-*CCHCR1* (non-risk allele or risk allele) constructs using FuGENE 6 reagent (Roche Diagnostics, Germany) according to the manufacturer's instructions. The native form of *StAR* in pcDNA3.1 without tags was also used in some assays. After transfection the cells were incubated in 1.5 ml media for 24 h or 48 h, after which media samples were collected for pregnenolone quantification. After the sample collection cells were cultured for an additional 24 h with 22R-hydroxycholesterol (1 µg/ml). In order to compensate for variation in transfection, efficiency assay results were normalized with pregnenolone concentrations produced by cultures with 22R-hydroxycholesterol.

Pregnenolone concentrations of media samples were determined by the LC-MS/MS system. Some results were also assayed for comparison with our radioimmunoassay method using liquid extraction and Lipidex-5000 microcolumn chromatography prior to RIA. Before LC-MS/MS analysis, 30 µl of 0.2 µM deuterated pregnenolone as internal standard (17,21,21,21-D4-pregnenolone, C/D/N Isotopes Inc., Pointe-Claire, Canada) in 50% (vol/vol) methanol was added to medium samples of 250 µl and samples were extracted with ether-hexane (90:10). Extracted samples were evaporated to dryness under nitrogen and dissolved in 250 µl of 50% methanol. Calibrators containing 1-100 nmol/l of pregnenolone (Sigma Chemical Co., St Louis, MO) were also prepared in 50% methanol. Twenty-five µl of sample extracts and calibrators were analyzed with an LC-MS/MS system equipped with an API 3000 triple quadrupole mass spectrometer (PE Sciex, Foster City, CA). Peripherals included an Agilent series 1100 HPLC system with a binary pump (Waldbronn, Germany). Separation was performed using a SunFire C18 column (2.1 x 50

mm; Waters, Milford, MA). The mobile phase was a linear gradient consisting of methanol and 100  $\mu$ M ammonium acetate in water, at a flow rate of 250  $\mu$ l/min. The gradient was: 0 min 50% methanol; 2 min 95% methanol; 3 min 95% methanol; and 3.5-10 min 50% methanol. The column was directly connected to the electrospray ionization probe. Pregnenolone was detected as ammonium adduct in the positive mode with the following transitions: m/z 334 to m/z 281 and IS, m/z 338 to m/z 285. Data were acquired and processed with the Analyst Software (Ver 1.3; Sciex). The mass calibration and resolution adjustments (at 0.7 atomic mass units at full width and half height) on both the resolving quadrupoles were optimized using a polypropylene glycol solution with an infusion pump.

## **5. Statistical analysis (I, II, III)**

Statistical analyses were made using Microsoft Office Excel. Statistical comparisons between data sets were performed with Student's t-test.

## RESULTS

### 1. Transgenic CCHCR1 mice

#### *1.1 Generation of transgenic mice (I)*

Transgenic CCHCR1 mouse models were engineered in order to gain more information about the function of CCHCR1. As CCHCR1 is expressed in keratinocytes, both in normal and psoriatic skin, K14 promoter targeting the transgene expression, predominantly to basal epidermal keratinocytes and outer root sheath of hair follicles, was used. We also wanted to study whether the function of the psoriasis-associated risk allele CCHCR1\*WWCC is different when compared to the common low-risk allele. For that purpose we made two constructs where either the wild-type or the psoriasis-associated form of human CCHCR1 cDNA was placed under the K14 promoter (I, fig 1A). Multiple lines of transgenic mice were generated and the incorporation of the transgenes was assured by Southern blots and PCR analyses from the genomic murine DNA. Analyses showed successful transgene incorporation in 14 normal allele mice out of 81 mice born and in 17 risk allele mice out of 75 born mice. All transgenic mice were viable and fertile and did not show any obvious phenotypic abnormalities (I, fig 1B). Two normal allele and three risk allele mice were used as founder animals and bred with the FVB/N strain to generate positive CCHCR1 mice lines.

#### *1.2 Characterization of the transgene CCHCR1 expression (I)*

In addition to checking the incorporation of the transgenes in DNA, also their expression was studied. Transgene expression on mRNA and protein levels were confirmed by RT-PCR and western blotting from the skin samples. All the transgenic lines overexpressed human CCHCR1 both on mRNA and protein level (I, fig 1C). We also measured transgene expression levels with quantitative RT-PCR (TaqMan). To facilitate comparisons between the transgenic lines, the acquired expression levels were normalized with the lowest expression value (expression of line 132 was set for 1). Normalized expression values for non-risk lines 12 and 34 were  $3 \pm 1$  and  $8 \pm 6$  and for risk allele lines 106, 128 and 132  $1.2 \pm 1.7$ ,  $13 \pm 2.8$  and  $1 \pm 0.6$ , respectively. The variability of the expression levels in one mouse line may partially be explained by the sporadic expression of CCHCR1 in epidermis. The copy doses of transgenes in different mouse lines were also calculated from the

intensities of the Southern blot bands. The data agreed with the TaqMan-based expression values with one exception: line 128 with the highest mRNA expression level seemed to have less copies of transgene than lines 12 and 34.

### *1.3 Histological evaluation of the transgenic CCHCR1 mice (I)*

All transgenic mice were healthy and viable and based on a routine health surveillance mice were free from ectoparasite infections. According to PAS stainings, the mice did not have fungal colonization either. The skin was morphologically grossly normal and indistinguishable between the transgenic and control mice as studied with hematoxylin-eosin staining (I, Fig. 2A-C). Human CCHCR1 expression was detected from basal keratinocytes (I, fig. 2D and E). This is in agreement with the K14 promoter-targeted expression. Immunostainings with frozen sections did not reveal additional CCHCR1 staining when compared to paraffin sections. Wild-type mice were negative with the antibody against human CCHCR1 as expected. There were differences in the immunostaining intensities between the transgenic lines as well as between the mice belonging to the same line. The observation was in agreement with the quantitative RT-PCR data. CCHCR1 homozygous mice were also morphologically normal. There were no obvious differences between the transgenic and wild-type mice stainings with commonly used psoriasis markers including proliferation marker Ki67, the differentiation marker cytokeratin 5 and the T-lymphocyte marker CD3. Taken together, transgenic CCHCR1 mice appeared healthy and there were no differences or features typical for psoriatic skin observed in the mice.

### *1.4 Gene expression profiles of microarray analyses (I)*

To understand the possible biochemical consequences of CCHCR1 expression in the skin, we studied gene expression profiles of the transgenic mice with Affymetrix expression analysis. mRNA samples from four risk, four non-risk and three wild-type mice were included in the analysis. The transgenic mice were from four different mouse lines and their transgene expressions levels did not differ significantly. Differentially expressed genes between the different mice groups were identified based on a combination of >2-fold expression changes and t-tests ( $p < 0.001$ ). We also considered whether the means of gene expression in any two groups were significantly different. In order to verify the Affymetrix data, expression of selected genes, namely S100A10, krt-6 and mmp9, were analyzed with

quantitative RT-PCR. The results for these selected genes were congruent with the Affymetrix data (I, fig. 3D). Otherwise expression results presented are based on the Affymetrix data.

#### 1.4.1 Differentially expressed genes between transgenic mice and wild-type mice (I)

When the gene expression of transgenic and wild-type mice were compared, a number of significant differences were observed, as 43 genes were at least four-fold upregulated and 63 genes were at least four-fold downregulated in the transgenic CCHCR1 mice (I, Table 1). A number of these genes have a role in skin, and among the most interesting genes are matrix metalloproteinases 3 and 13 (Mmp3, 13), betacellulin (Btc), tumor necrosis factor receptor superfamily member 6 (Tnfrsf6), alpha-induced protein 2, procollagen, type III, alpha 1 (Col3a1) and calmodulin 4 (Calm4). The gene showing the most consistent upregulation in all 8 transgenic mice was keratinocyte differentiation association factor (Krt dab) with almost four-fold increase in expression. Several keratin-associated proteins and hair keratins, S100 Ca-binding protein A3 (s100a3) and fibroblast growth factor 5 (Fgf5) were highly significantly downregulated in transgenic mice. Among less significantly downregulated genes were cathepsin E, small proline-rich protein 2H (sprr2H) and desmocollin 2.

#### 1.4.2 Differential expression between transgenic lines (I)

To highlight the functional differences caused by CCHCR1\*WWCC risk allele in comparison to normal CCHCR1 allele, expression profiles between the two transgenic mice group were compared.

Fifty-six genes were at least two-fold and 14 genes at least three-fold upregulated in risk mice as compared to non-risk mice (I, table 2). The highest upregulations were seen with several small proline-rich proteins (1B, 2A and 2D), keratin 6a (Krt2-6a) and its partner keratin 16 (Krt1-16) and Janus kinase 2 (Jak2). Less upregulated genes included for example tenascin C, interleukin 6, Caspase 6 and small proline-rich proteins 2H and 2F (Sprr2H, 2F). Among interesting genes shown to be upregulated by t-test-based analyses ( $p < 0.001$ ) were trans-acting transcription factor 4 (Sp4), small proline-rich proteins 2F and 1B (Sprr2F, 1B), zinc finger protein regulator of apoptosis and cell cycle arrest (Zac1), defensin beta1 (Defb1) and keratin 6a (Krt2-6a).



Also a significant number of genes were downregulated in the risk mice as compared to non-risk mice: 83 genes were downregulated at least two-fold and 16 genes showed at least three-fold difference in expression (I, table 2). Among the downregulated genes were several keratin-associated proteins and acidic hair keratin 5 (Krt1-24). Among less significantly downregulated genes were type I hair keratin (Krt1-1), basic hair keratin 5 (Krt2-18), cytokeratin 19(Krt1-19), matrix metalloproteinase 12 (Mmp12) and S100 Ca binding protein A3 (s100a3). With the t-test, the following genes showed significant downregulation ( $p < 0.001$ ): cadherin 3 (Cdh3), AP-1 beta subunit (Ap1b1), S100 Ca binding protein A13 (s100a13), cadherin 1 (Cdh1), IL-6 signal transducer (Il6st), ubiquitin C (Ubc) and S100 Ca binding protein A10 (s100a10).

#### 1.4.3 Pathway and cluster analysis of gene expression differences (I)

The changes in the expression of the genes participating in different psoriasis-associated signaling pathways were studied with Biocarta net tools. Signaling pathways studied included NF- $\kappa$ B signaling, keratinocyte differentiation, EGF, TGF- $\beta$  and IFN- $\gamma$  signaling pathways, cytokines and inflammatory response, cell-to-cell adhesion, and cytoskeleton organization and biogenesis. With t-test value  $p < 0.01$ , significant changes were detected in the cytoskeleton organization and biogenesis pathway, in which keratins 6a (Krt2-6a) and 16 (Krt1-16) and alpha 2 actin were upregulated in the risk mice compared to non-risk mice.

Similarity of gene expression patterns was studied by cluster analyses. In addition to the usage of the entire expression data, keywords such as integrin, interleukin, transcription factor, proteinase, binding protein, protease or keratin were used for subgrouping the data for the cluster analyses. To exclude possible differences caused by hormonal background, we limited analyses to male mice only. Among seven two-way cluster tree studies, only the keratin group with 57 transcripts, representing 37 genes, showed clearly distinct gene clusters and the separation among risk, non-risk and wild-type mice was pronounced (I, fig. 3). Keratin-related genes were divided into two main groups: 20 genes showed higher expression levels in risk mice when compared to the non-risk or wild-type mice, and 14 genes showed lower expression levels in risk mice. One interesting gene is keratinocyte differentiation-associated protein (Krtdap), which was differentially expressed according to the CCHCR1 genotype. It was upregulated in risk mice as compared to non-risk mice and downregulated in wild-type mice. Keratin 5, keratin 6a and keratin 17 expression was

higher in risk mice as compared to either non-risk or wild-type mice. Most genes showing lower expression level in risk mice belonged to hair keratins or keratin-associated proteins expressed mainly in hair. The independent clustering profiles of keratin-related genes, between the risk and other mice types, suggest the importance of these genes in classifying the different mouse groups and it also promoted the distinct functional aspects between the normal and psoriasis-associated CCHCR1 alleles.

## **2. Functional Studies of CCHCR1**

### *2.1 CCHCR1 localization in cells (II)*

The cellular localization of both normal and psoriasis-associated forms of CCHCR1 was studied in transfected cells. Both forms of CCHCR1 showed punctate staining signals in the cytoplasm (II, fig. 3a and d). The exact nature of the CCHCR1-containing structures remained unclear. CCHCR1 did not co-localize with the endosomal proteins, including early endosomal autoantigen 1, the late endosomal protein MLN64 or transferrin receptor which localizes to recycling compartments, or with the lysosomal marker Lamp1 or mitochondria (II, fig. 3i). In an experiment with a fluid-phase tracer, CCHCR1-GFP positive structures were not accessible to endocytosed dextran. As CCHCR1 was shown to play a role in steroidogenesis, we also studied whether CCHCR1 was present in lipid droplets. As studied by the neutral lipid stain Nile Red, CCHCR1 did not co-localize with lipid droplets (II, fig. 3l). In addition, the CCHCR1 structures did not sequester either with filipin, indicating distribution of free cholesterol or NBD-cholesterol detecting the lipid droplets, suggesting that CCHCR1-containing particles were not enriched with unesterified cholesterol. We did not observe any obvious differences between the non-risk and risk forms of CCHCR1.

### *2.2 CCHCR1 regulation studies (II)*

We also studied the expression levels of CCHCR1 in keratinocytes derived both from healthy skin ( $n = 3$ ) and non-lesional psoriatic skin ( $n = 4$ ) (Karvonen et al. 2000) with quantitative real-time PCR (TaqMan). Interestingly, the expression of CCHCR1 was downregulated two-fold in non-lesional psoriatic keratinocytes as compared to normal keratinocytes ( $p < 0.05$ ), suggesting the dysregulation of CCHCR1 in psoriatics (II, fig.

5a). Expression levels of CCHCR1 did not differ according to the CCHCR1 genotype, but the sample size was too small to draw firm conclusions on genotype specific-effect.

In addition, we wanted to study the regulation of CCHCR1 expression. For that purpose we treated HaCaT keratinocytes with several growth factors, hormones and anti-psoriatic agents and measured the mRNA expression levels with quantitative real-time PCR. The highest change in the expression level with almost three-fold upregulation was observed after insulin treatment. Estrogen and forskolin (with p-value 0.06) also upregulated CCHCR1 by 50%, whereas the immunosuppressant cyclosporin A downregulated CCHCR1 expression by two-fold. Dihydrotestosterone (Stanolone) or thyronine did not affect CCHCR1 expression on mRNA levels (II, fig. 6b). The effect of epidermal growth factor (EGF) on CCHCR1 expression on the protein level was also investigated. The level of endogenous CCHCR1 protein in HaCaT cells was too low to be detected by western blotting from total cell lysate, but after treatment with EGF, a faint band corresponding to the size of CCHCR1 was observed, suggesting EGF as a stimulator of CCHCR1 expression (II, fig. 6c). Interestingly, cholesterol metabolism also affects CCHCR1 expression. When cholesterol was depleted from HaCaT keratinocytes by cholesterol extraction with 7.5 mM methyl- $\beta$ -cyclodextrin (M $\beta$ CD) and neosynthesis inhibition with 10  $\mu$ M lovastatin (Jans et al. 2004) CCHCR1 expression showed five-fold downregulation (II, fig. 6a). The downregulation was smaller if the neosynthesis was not prevented.

### *2.3 The role of CCHCR1 in keratinocyte proliferation and migration*

#### *2.3.1 Wound healing in transgenic mice expressing CCHCR1 (III)*

Wounding can trigger formation of a psoriasis plaque according to Koebner's phenomenon and it has also been shown to cause psoriasis-like phenotypes in transgenic psoriasiform mouse models (Sano et al. 2005a; Zenz et al. 2005). To study the effects of wound healing in the bitransgenic CCHCR1 mice, circular full-thickness 5 mm diameter wounds on the dorsal skin on both wild-type and transgenic CCHCR1 mice overexpressing either healthy or psoriasis-associated alleles of human CCHCR1 were conducted. Mice were sacrificed 1, 4, 11 and 30 days post-wounding, the wound areas were measured and wound samples were collected for further analyses so that 8 wounds from each mouse group were studied at each time point. Measurements showed that early

wound closure at days 1 and 4 post-wounding was delayed in risk mice when compared to wound closure in non-risk mice (day 1,  $p < 0.01$  and day 4,  $p < 0.05$ ) (III, fig. 1A). Macroscopic measurement results were supported by histological evaluation and measurement of the newly formed epithelium (III, fig. 1B, C and D). Interestingly, the epithelial layer was still absent from the wounds in risk mice 4 days post-wounding, whereas in non-risk mice, neoepidermis was visible already on day 1 and 50 % of the wounds were completely re-epithelialized on day 4. Wound closure and regeneration of epidermis was completed in all mouse groups at day 11. No clinical or histological features of psoriasis development were observed in the wounded areas during the 30 days observation period.

To determine the underlying factors causing the delayed wound closure in risk mice, wounds were histologically evaluated. Evaluation of dermal components according to the modified Greenhalg criteria (Spenny et al. 2002; Puolakkainen et al. 2005) did not indicate any abnormalities in inflammation, formation of granular tissues or neoangiogenesis. As these results rule out the dermal components as the primary defect causing the delayed wound closure, they suggest that the primary cause of retarded healing might be impaired regeneration of the epidermis, which involves proliferation and migration of keratinocytes. The migratory properties of keratinocytes were studied with laminin-5 staining (Pyke et al. 1994) and the apoptosis with TUNEL staining, but they failed to show differences between the mice groups. Neither staining of mast cells nor immunohistochemical analyses of hyperproliferation marker cytokeratin 6, myofibroblast marker  $\alpha$ -smooth muscle actin and von Willebrand factor, a marker of neoangiogenesis, did reveal any abnormalities in transgenic mice. Staining with the proliferation marker Ki67 showed that one day after wounding only a few proliferating keratinocytes were found at the wound edges and there were no differences between the mouse groups. However, on day 4 the risk mice had significantly fewer proliferating cells in newly formed epidermis than wild-type or non-risk mice ( $p < 0.001$ ) (III, fig. 1E). A similar effect was also seen 11 days post-wounding ( $p=0.01$ ) and the same trend was seen even 30 days after the wounding. This highly significant difference suggested impaired keratinocyte proliferation as the underlying defect causing delayed wound closure in transgenic mice with CCHCR1\*WWCC risk allele.

### 2.3.2 Single dose TPA application of transgenic CCHCR1 mice (III)

12-O-tetradecanoylphorbol-13-acetate (TPA) is a chemical used to trigger psoriasis-like phenotype in mouse models (Sano et al. 2005a). In order to induce epidermal hyperplasia, the dorsal skin of transgenic CCHCR1 and wild-type mice were treated once with topical TPA application. Skin samples for histological analyses were collected 24, 48 and 72 h after the treatment. All mouse groups showed epidermal thickening 24 h after the treatment. However, there were interesting differences between the mouse groups as the TPA-treated transgenic CCHCR1 mice showed a less well-developed epidermal hyperplasia than the wild-type controls, and the difference was most evident 48 h after the treatment when the number of proliferating cells, as indicated with Ki67 staining, was 20-30% lower in transgenic mice than in wild-type mice (III, fig. 2A and B). The CCHCR1\*WWCC allele mice had 14 % fewer proliferating cells than the non-risk mice ( $p < 0.05$ ) (III, fig. 2D). The location of proliferating cells was also different, in transgenic mice proliferating cells were confined mainly to the basal layer in epidermis, whereas in wild-type mice proliferation was observed also suprabasally (III, fig. 2C). There were no differences in keratinocyte differentiation between the mouse groups as indicated by the uniform expression of the differentiation marker cytokeratin 10, and also the number of infiltrating T cells was indistinguishable between the mouse groups.

### 2.3.3 Cell proliferation in untreated mouse skin and cultured keratinocytes of CCHCR1 mice (III)

In addition to the study of CCHCR1 functional aspects in response to different kinds of treatments, we wanted to study the effects of CCHCR1 on normal keratinocyte proliferation. To study cell proliferation, bromodeoxyuridine (BrdU) was injected intraperitoneally into the wild-type and transgenic CCHCR1 mice, skin samples were collected after an incubation period and BrdU-labeled cells were detected by immunohistochemistry. Interestingly, after 6 h labeling time the proportion of BrdU positive cells was 63% in the risk allele mice and 77% and 73% in the non-risk allele and wild-type mice, respectively. Observed differences suggested impaired cell proliferation in risk mice as compared to the non-risk mice ( $n=4$ ,  $p < 0.05$ ) (III, fig. 3). A similar trend was also seen with Ki67 staining of transgenic mice skin samples ( $n=3$ ).

The impact of transgenic CCHCR1 and its different gene forms on the proliferation rate in response to EGF was studied also in cultured keratinocytes derived from newborn transgenic CCHCR1 or wild-type mice. Cells were labeled at different time points with BrdU in the presence or absence of EGF. The amount of incorporated BrdU was determined with ELISA. The measured absorbance values were plotted as a function of time, and relative proliferation rates were estimated from the slope of the line (III, fig. 3B and C). The relative proliferation rate was slower in keratinocytes expressing the risk allele of CCHCR1 when compared to wild-type keratinocytes, both in the presence or absence of EGF. However, no obvious difference in proliferation rate between cells expressing the risk allele and non-risk allele of CCHCR1 was observed.

In addition to the effects of CCHCR1 and its different genotypes on proliferation, we have also studied keratinocyte migration with newborn transgenic and wild-type mice. Migration properties were studied with the Transwell chambers or an *in vitro* scratch assay (Matthay et al. 1993; Cha et al. 1996). We also used a recently described skin explants model to study the radial outgrowth of mouse epidermis (Lu&Rollman 2004; Mirastschijski et al. 2004). These experiments showed that the different alleles of CCHCR1 did not affect the migration of keratinocytes.

## 2.4 CCHCR1 in steroidogenesis

### 2.4.1 Expression of human StAR in skin (II)

CCHCR1 has been shown to induce steroidogenesis by interacting with the StAR protein (Sugawara et al. 2003). Even though skin is defined as a steroidogenic tissue, the expression of StAR protein has not been reported in skin. We studied StAR expression with RT-PCR from cultured human primary keratinocytes and HaCat cells and interestingly, StAR mRNA was observed from both cell types (II, fig. 1a). StAR protein expression was detected in normal healthy epidermis by immunostaining (II, fig. 1a) and its expression was confined to basal keratinocytes, in the same area as CCHCR1 expression (II, fig 1b). In non-lesional psoriatic skin these two proteins were expressed in the basal keratinocytes (II, fig. 1e and f). CCHCR1 and StAR were expressed in the same regions in various steroidogenic tissues, including testis and adrenal glands (II, fig. 1g, h, I and j). In lesional psoriatic skin, CCHCR1 and StAR expression was detected only in partially overlapping areas as StAR expression was most abundant in basal keratinocytes

of the rete ridges (II, fig 1c) and CCHCR1 was expressed in keratinocytes above dermal papillae (II, fig. 1d; Asumalahti et al. 2002; Suomela et al. 2003). However, when the localization of CCHCR1 and StAR was studied in transfected cells, surprisingly only a minority of CCHCR1 signals was overlapping with StAR protein (II, fig. 3). The StAR antibody used did not cross-react with the StAR homolog MLN64, as indicated by the negative placenta staining (Stocco et al. 2005). In conclusion, according to our study, StAR is expressed in the same areas as CCHCR1 in normal human skin and steroidogenic organs and the expression pattern is disturbed in lesional psoriatic skin where they show an inversed expression pattern.

The expression level of StAR and P450scc was also studied in both healthy and non-lesional cultured primary human keratinocytes. Both genes were expressed in keratinocytes (II, fig. 5b) and there was no difference in the expression level between keratinocytes derived from healthy or psoriatic persons. Interestingly, P450scc upregulated five-fold after cAMP-mediated stimulation caused by forskolin, whereas the StAR expression level remained unchanged (II, fig. 5b). This supported the previous observation about the cAMP-independent regulation of StAR expressions observed in other steroidogenic tissues to be valid also in keratinocytes (Mamluk et al. 1999).

#### 2.4.2 Functionality of CCHCR1 in steroidogenesis (II)

CCHCR1 has been shown to enhance pregnenolone production in cultured cells (Sugawara et al. 2003). To further study the function of CCHCR1 and its psoriasis-associated CCHCR1\*WWCC form in steroidogenesis, COS-1 cells were transfected with the F2-system encoding other enzymes needed for the reaction (Pollack et al. 1997), StAR plasmid and the risk or non-risk form of *CCHCR1*. The amount of pregnenolone produced by COS cells was determined by mass spectrometry. Interestingly, during the 24 h and 48 h sampling periods, the risk form of CCHCR1 enhanced pregnenolone production as well as the non-risk CCHCR1 (II, fig 2). The increment in the amount of pregnenolone was similar to that reported previously for CCHCR1 (135%) (Sugawara et al. 2003).

#### 2.4.3 Steroidogenic genes in transgenic CCHCR1 mice (II)

The previous Affymetrix expression profiles of genes related to steroid or sterol metabolism were further analyzed. A comparison of 8 transgenic mice (including both

genotypes) and 3 wild-type mice showed 3 relevant genes with clear differences in the expression. The most significant change with a 3.8 fold downregulation in transgene mice was observed in the expression of vitamin D receptor gene (Vdr). In addition, two-fold downregulation in transgenic mice with less significance was observed for NaD(P)-dependent steroid dehydrogenase (Nsd1) and sulfotransferase 2B1 (Sult2B1).

## *2.5 CCHCR1 and Vitamin D (II)*

As the Vdr gene was differently expressed between transgenic and wild-type mice in previously conducted Affymetrix analyses, we wanted to study the expression of vitamin D receptor more carefully. Vdr expression was also almost four-fold downregulated in cultured keratinocytes derived from risk mice when compared to the Vdr expression in non-risk mice (II, fig. 4). We also studied the expression of human VDR in non-lesional psoriatic keratinocytes. Interestingly, the VDR gene expression was downregulated in non-lesional psoriatic keratinocytes four-fold at mRNA level as compared to normal keratinocytes ( $p = 0.01$ ) when analyzed by quantitative RT-PCR (II, fig. 5a). The CCHCR1 genotype did not have any obvious effect on VDR expression level.



## DISCUSSION

Even though psoriasis is a common skin disease affecting more than 2% of the population, its molecular level pathogenesis is still poorly understood. A main problem in psoriasis research is its multifactorial etiology with multiple genes involved. The role of PSORS1 in the etiology of psoriasis is without doubt and is the strongest genetic determinant of psoriasis. Despite the convincing amount of research, the identity of the PSORS1 gene still remains unknown and the debate about the exact genomic localization of PSORS1 gene, and the presence or absence of the candidate genes HLA-C, CDSN and CCHCR1 at the most significant area, has been intense (Asumalahti et al. 2000; Nair et al. 2000; Veal et al. 2002; Holm et al. 2003; Helms et al. 2005; Chang et al. 2006; Zhang et al. 2009). A recent large genome-wide association study located the PSORS1 gene to the region flanked by HLA-C and CCHCR1 gene ( $P < 10^{-150}$ ) and according to the study, CDSN is outside the area of strongest association (Zhang et al. 2009). This study is one of the first PSORS1 studies conducted with non-Caucasian populations and it interestingly transfers the area of the strongest PSORS1 association towards CCHCR1, away from HLA-C. Another noteworthy fact is that of the SNPs showing the best association, only one is coding, and it is the SNP coding the first arginine tryptophan change in the CCHCR1\*WWCC allele. In addition to the uncertainty of the PSORS1 gene, the lack of good mouse models has also hindered psoriasis research, although recently many good mouse models representing notable numbers of psoriatic features have been introduced.

### 1. Gene expression profile of CCHCR1 mice

The role of CCHCR1 and its psoriasis-associated allelic form CCHCR1\*WWCC was studied in transgenic mice overexpressing both CCHCR1 gene forms under the K14 promoter, targeting expression to basal keratinocytes. Skin of the mice was normal and indistinguishable between the two transgenic mouse groups. However, gene expression profiling suggests that CCHCR1 affects the expression of numerous genes relevant for psoriasis and showing altered gene expression in human psoriatic skin. Mouse models also suggested that CCHCR1 has allele-specific effects on the expression of genes implicated in psoriasis. For example, cytokeratins 6 and 16 which are upregulated in human psoriasis (Bowcock et al. 2001) were also upregulated in risk allele mice as compared to non-risk mice. The highest upregulation in transgenic risk allele mice was observed for small

proline-rich proteins (Sprr) 1B, 2A and 2D. SPRRs are precursor proteins for the cornified envelope (Backendorf&Hohl 1992) which are induced during the keratinocyte differentiation, and for example, SPRR2A and 1B are upregulated in human psoriatic skin (Bowcock et al. 2001). The localization of the human SPRR gene cluster in the PSORS4 locus also makes them interesting in the context of psoriasis. Interestingly, members of the S100 protein family, including S100a3, a10 and a13, were downregulated in risk allele mice. The S100 genes are calcium-activated signaling proteins known to be involved in keratinocyte differentiation and the pathogenesis of psoriasis (Bowcock et al. 2001; Broome et al. 2003; Nukui et al. 2008). Interestingly, a number of human S100 genes are also located in the PSORS4 locus. Members of both S100 and SPRR gene families are regulated via Jun/AP-1 signaling, which make them even more relevant for the pathogenesis of psoriasis, as the Jun signaling is an important regulator of keratinocyte differentiation and proliferation (Zenz&Wagner 2006). The allele-specific effects of CCHCR1 were also highlighted by the cluster analysis in which the keratin-related genes showed remarkable genotype-specific expression profiles: the profile of risk mice diverged clearly from the profile of non-risk mice, which for one showed high similarity to expression profiles of wild-type mice. Interestingly, “keratin” was the only keyword showing allele-specific expression patterns as for example “integrin”, “interleukin” or “transcription factor” failed to show any allele-specific patterns. Transgenic mice overexpressing the psoriasis-associated or the non-risk form of human CCHCR1 were the first mouse models for a psoriasis susceptibility gene mapping to a confirmed locus. In addition, they are the first models so far expressing, in addition to the non-risk human gene form, also the psoriasis-associated form of a putative susceptibility gene

## **2. The role of CCHCR1 in proliferation**

As the skin of the transgenic mice was indistinguishable from wild-type mouse skin, we wanted to test if introducing additional environmental challenges, including wounding and TPA treatment, would give rise to psoriasis-like features or differences between the mouse groups. CCHCR1 mice did not develop characteristics typical for psoriasis but the results obtained from the experiments suggested that CCHCR1 may play a role in keratinocyte proliferation. Early wound closure was delayed in the risk mice (III). The mechanism for the impaired wound healing was the smaller number of proliferating keratinocytes in risk mouse epidermis as compared to either the normal transgenic or wild-type mice. The

response to TPA-induced hyperplasia was also impaired in risk allele mice as all the mice groups responded to TPA, but the hyperplasia was less well-developed in risk mice and they also had fewer proliferating cells. As the CCHCR1\*WWCC allele mice showed impaired keratinocyte proliferation in response to external stimuli, also its possible effect in untreated mice was studied. Indeed, CCHCR1\*WWCC affected keratinocyte proliferation also in native circumstances as revealed by *in vivo* BrdU labeling of untreated mice: risk allele mice had less proliferating keratinocytes than non-risk or wild-type mice. One can conclude that the overexpression of CCHCR1\*WWCC resulted in reduced keratinocyte proliferation, which is highly relevant in the context of the pathogenesis of psoriasis.

A potential role in one of the most important aspects of psoriasis pathogenesis is highly interesting and offers a possible mechanism for CCHCR1 to impact the development of psoriasis. In addition to experimental mouse data, other properties of CCHCR1 also supported its role in the regulation of keratinocyte proliferation. We have shown in our earlier studies that CCHCR1 is expressed at the sites that are less active for cell proliferation in psoriatic skin (Asumalahti et al. 2002; Suomela et al. 2003). A similar phenomenon has also been seen in breast and lung carcinomas in which CCHCR1 was expressed in nonproliferating cells (Suomela et al. 2003). The amount of CCHCR1 protein may also be critical for the proliferation and pathogenesis of psoriasis. This is supported by the observation that CCHCR1 expression was downregulated in cultured non-lesional psoriatic keratinocytes when compared to healthy keratinocytes. However, this downregulation has not been observed in our previous *in vivo in situ* experiments with lesional psoriatic skin. This may reflect differences between *in vivo* and *in vitro* experiments. Furthermore, the samples were different as the *in situ* results were gained from lesional psoriatic skin and the qRT-PCR analyses were done with cultivated non-lesional keratinocytes. Also the altered expression of the psoriasis hyperproliferation markers K6, 16 and 17 in transgenic CCHCR1 risk mice, as compared to either non-risk or control mice, shown by expression profiling, supports the potential involvement of CCHCR1 in cell proliferation (I). Our regulation studies also supported the CCHCR1 involvement in proliferation, as agents involved in the regulation of proliferation, including interferon  $\gamma$ , insulin, EGF and estrogen, affected the levels of CCHCR1 expression (II; Suomela et al. 2003) .

### 3. CCHCR in steroidogenesis

CCHCR1 has been shown to promote steroidogenesis via interaction with the StAR protein (Sugawara et al. 2003) and based on our immunohistological localization data, this may also occur in skin. We also demonstrated that the psoriasis risk allele showed similar activity in steroidogenesis as the non-risk form. Steroid hormones have a number of important functions in skin, of which many are highly relevant also in the context of psoriasis. Steroid hormones are required for cell proliferation, epidermal barrier homeostasis, differentiation and modulation of immune response (Zouboulis&Degitz 2004; Bikle et al. 2005). Interestingly, some skin diseases are also directly caused by inborn errors of cholesterol or steroid metabolism. For example, X-linked ichthyosis (OMIM 08100), characterized by epidermal hyperkeratosis, is caused by mutations in the steroid sulfatase gene (Shapiro et al. 1989). Steroid hormones can also have an effect on the disease course of skin diseases, as for example estrogen modulates symptoms of psoriasis (Kanda&Watanabe 2005; Murase et al. 2005). Skin and keratinocytes are actively involved in steroidogenesis, as all the essential enzymes and cofactors of the steroidogenic cascade are present in skin (Venencie et al., 1999; Thiboutot et al. 2003).

Steroid hormones are synthesized from cholesterol in the mitochondria. The first step of the synthesis is the conversion of cholesterol to pregnenolone by the cytochrome P450 side chain cleave (P450scc) enzyme in the inner mitochondria membrane. Pregnenolone is further converted to progesterone and then to a variety of other steroids. The spectrum of the final products is defined by the tissue specific enzyme assortment. StAR protein participates in and regulates the transportation of cholesterol from the outer mitochondria membrane to the inner membrane to be available for P450scc. StAR is expressed in steroid-producing tissues (Stocco et al. 2005) and interestingly according to our results, StAR is expressed also in the basal keratinocytes of the skin. In healthy human epidermis, StAR expression was confined to the areas where also CCHCR1 expression was most abundant. StAR expression was altered in psoriatic skin and the expression pattern also varied between the non-lesional and lesional psoriatic skin. In non-lesional psoriatic skin, StAR expression was inverse compared to CCHCR1 expression. In addition to interaction with StAR, agents relevant in steroidogenesis affected the expression of CCHCR1, supporting the connection between CCHCR1 and steroidogenesis. Interestingly, cholesterol, the substrate of steroid hormones, affected the expression of CCHCR1 in keratinocytes, as the

amount of CCHCR1 decreased 5-fold after cholesterol depletion. Deficient cholesterol metabolism causes alterations to keratinocyte differentiation. Furthermore, cholesterol depletion influences the expression levels of a variety of differentiation marker genes (Jans et al. 2004). In addition to cholesterol, other factors also relevant in steroidogenesis influence CCHCR1 expression. For example, epidermal growth factor (EGF) and insulin-like growth factor (IGF) are known activators of steroidogenesis (Stocco et al. 2005) and also stimulate CCHCR1 expression. Also, the steroid hormone estrogen stimulates CCHCR1 expression as well as forskolin, a known steroidogenesis activator. Based on the StAR interaction and expression data, it appeared well justified to conclude that CCHCR1 is connected to steroidogenesis. Skin is a steroidogenic tissue and thus able to produce steroid hormones locally. Locally produced steroids can be highly important for psoriasis as they may participate, for example, in the regulation of cell proliferation, differentiation and immune response (Bikle et al. 2005; Lehmann 2005). Defects in steroidogenesis are likely to impact on the pathogenesis of psoriasis as indicated also by the altered StAR expression observed in psoriatic skin. As CCHCR1 promotes steroidogenesis and the knockdown of CCHCR1 with RNA interference reduces the amount of pregnenolone in cultured steroidogenic cells (Sugawara et al. 2003), CCHCR1 seems to have a definite role in the synthesis of steroid hormones. CCHCR1 may influence steroidogenesis via regulation of StAR. The low CCHCR1 level observed in psoriatic keratinocytes may also be relevant, as the amount of CCHCR1 may affect the efficiency of steroidogenesis. Taken together, it is reasonable to conclude that CCHCR1 may influence local steroidogenesis in skin and by that it may affect processes relevant for the pathogenesis of psoriasis.

Our results suggest that CCHCR1 may also have a role in skin vitamin D metabolism, which is highly interesting in the context of psoriasis. Vitamin D is a steroid hormone that regulates keratinocyte proliferation and differentiation, as well as formation of the epidermal barrier (Reichrath 2007). It is also successfully used as a treatment for psoriasis (Schauber&Gallo 2008). In addition, vitamin D receptor (VDR) polymorphisms have been suggested to associate with psoriasis (Saeki et al. 2002; Halsall et al. 2005; Dayangac-Erden et al. 2007) and the level of VDR has been proposed to affect the responsiveness to vitamin D therapy in psoriasis patients (Carlberg&Saurat 1996). According to our study, the vitamin D receptor is expressed by keratinocytes and its expression is decreased in non-lesional psoriatic keratinocytes. Also the *Vdr* expression in CCHCR1\*WWCC risk allele mice was downregulated when compared to transgenic mice with normal CCHCR1 or wild-

type animals. This genotype-specific effect suggested an impaired function for the CCHCR1 risk form in vitamin D metabolism, and as the level of VDR is known to affect the response to vitamin D treatment, this altered VDR level is important to consider.

Keratinocytes appear as a central cell type in skin steroidogenesis and vitamin D metabolism, as they express both StAR and P450<sub>scc</sub>, the key enzymes of steroidogenesis. In addition to cholesterol, P450<sub>scc</sub> enzyme also metabolizes vitamin D<sub>3</sub> and its precursor 7-dehydrocholesterol (7-DHC) found mainly in basal epidermal keratinocytes (Slominski et al. 2004). Indeed, keratinocytes are the only cell type that has the entire metabolic machinery to produce the active form of vitamin D from 7-DHC and in addition has receptors for it. The P450<sub>scc</sub>-metabolized vitamin D forms also affect keratinocyte differentiation and their effects are mediated via VDR.

#### **4. Biochemical pathways of CCHCR1 function**

CCHCR1 has a role in the proliferation of keratinocytes but the mechanisms still remain unknown. There are a couple of alternatives based on the other functional aspects of CCHCR1. One possible mechanism how CCHCR1 could influence keratinocyte proliferation involves interaction with RNA polymerase II subunit 3 (RBP3) (Corbi et al. 2005). RBP3 is a part of the RNAPII core enzyme, which is composed of at least 12 different subunits. RNAPII associates with several mediator proteins and general transcription factors to form the holoenzyme complex, the activity of which is tightly controlled by several auxiliary factors in order to confer its proper tissue-specific activity. Interestingly, CCHCR1 has been suggested to serve as a cytoplasmic docking site for RPB3, regulating its movement from the cytoplasm to nucleus (Corbi et al. 2005). In addition to other regulatory proteins, RBP3 also interacts with the activating transcription factor 4 (ATF4/CREB2), a member of the ATF family. ATF4 is able to form heterodimers with other members of the AP-1 family, including Jun and Fos proteins. Interestingly, Jun proteins, namely c-jun, JunB and JunD are among regulators of keratinocyte proliferation, differentiation and cytokine production. It is also noteworthy that Jun proteins have been implicated to have a role in the pathogenesis of psoriasis: expression of JunB, located to the PSORS6 locus, is greatly reduced in lesional psoriatic skin (Zenz et al. 2005) and elevated c-Jun levels have also been observed in psoriatic skin (Mehic et al. 2005). In addition, the double knock-out c-jun/JunB mice spontaneously develop psoriasis-like phenotype (Zenz et

al. 2005). Individual members of the AP-1 family have specific functions in cellular processes and also the target tissues affect the functional characteristics of AP-1-mediated activity (Angel et al. 2001). Jun proteins are activated by Jun-amino-terminal kinases (JNKs). The activated c-jun-containing AP-1 complex promotes cell proliferation by inducing transcription of positive regulators of cell cycle progression, including cyclin D1 and suppressing the negative regulators, such as tumor suppressor p53 production (Zenz&Wagner 2006). The AP-1 family has also been proposed to be involved in wound healing because of its rapid and transient expression kinetics and response to extracellular signals. AP-1 activity is supposed to be required under conditions where the balance of keratinocyte proliferation and differentiation is changed rapidly and temporally, as is the case in wound healing (Angel et al. 2001). As CCHCR1 has a clear antiproliferative effect, which was most prominent after wounding and TPA treatment, it is tempting to propose that CCHCR1 might influence AP-1 signaling through regulation of RPB3 protein. The regulation could be dysfunctional in psoriasis due to a lowered CCHCR1 amount, as we have shown to be in psoriatic keratinocytes, or the allele-specific alterations caused by the CCHCR1\*WWCC risk allele. Even though AP-1-mediated regulation is the most important regulator of keratinocyte proliferation, additional regulation pathways for the proliferation and pathogenesis of psoriasis also exist and their role in the context of CCHCR1 remains to be elucidated.

As CCHCR1 is known to activate steroidogenesis via interaction with the StAR protein, steroids also offer a possible route for CCHCR1 to influence proliferation. Steroids can modulate wound healing in human and mouse skin and may also affect keratinocyte proliferation (Urano et al. 1995; Ashcroft et al. 1997; Verdier-Sevrain et al. 2004; Gilliver et al. 2006; Gilliver&Ashcroft 2007). Furthermore, vitamin D, a steroid hormone, affects keratinocyte proliferation and wound healing (Gurlek et al. 2002; Gamady et al. 2003). The effect of CCHCR1 in keratinocyte proliferation may be explained by its role in steroidogenesis. This is supported by impaired vitamin D receptor expression in psoriatic skin and in CCHCR1\*WWCC mice. Interestingly, both vitamin D and estradiols are involved in the biology of AP-1, as they both induce the expression of c-fos and c-jun, and AP-1 is also a mediator of vitamin D-induced differentiation (Johansen et al. 2004; Verdier-Sevrain et al. 2004). As vitamin D and its receptor participate in many functions relevant in the context of psoriasis and CCHCR1 seems to have an allele-specific effect on the expression of vitamin D receptor downregulated in psoriatic skin, this offers an interesting

functional mechanism for CCHCR1 in the pathogenesis of psoriasis. Possible mechanisms could be the regulation of StAR or P450scc, as those are suggested to metabolize vitamin D and its precursors from 7-DHC, and StAR is known to interact with 7-DHC (Slominski et al. 2004).



## CONCLUSIONS AND FUTURE PROSPECTS

CCHCR1 seems to affect keratinocyte proliferation and steroidogenesis, functions possibly overlapping. Both functions are highly important in the context of the pathogenesis of psoriasis. Furthermore, transgenic CCHCR1 mice support a role for the psoriasis-associated allele CCHCR1\*WWCC in the pathogenesis of psoriasis as the mice with the risk allele showed many allele-specific differences in processes relevant for psoriasis, including expression of genes relevant in psoriasis. The downregulation of CCHCR1 in human psoriatic keratinocytes for one suggests the possibility that the impaired function of CCHCR1 might be explained by its insufficient amount in psoriatic skin. There are tempting alternatives for potential mechanisms of CCHCR1 function mediated by AP-1 signaling steroids. However, the more detailed function of CCHCR1 and the mechanism, in which it participates, needs to be elucidated in more detail in the future.

It would be highly interesting to study if the amount of CCHCR1 impacts on its cellular functions. The possible effects of CCHCR1 inhibition also deserve to be investigated. In addition, as the cellular localization of CCHCR1 still remains unknown, it should be given attention in future studies. The research clarifying the allele-specific impact of CCHCR1 risk and non-risk forms is also highly relevant in order to understand the possible role of CCHCR1 in the pathogenesis of psoriasis.

PSORS1 is the major psoriasis susceptibility locus and transgenic CCHCR1 mice were the first transgenic mouse model for a candidate gene located in any psoriasis susceptibility locus. In addition, CCHCR1\*WWCC risk mice are so far the only transgenic mice targeting the psoriasis-associated allelic form of a psoriasis candidate gene. CCHCR1 mouse models offer an interesting view on the possible role of CCHCR1. However, there are matters to take into account concerning the different aspects of the model. The expression level of mouse endogenous CCHCR1 is not known, which makes it difficult to evaluate the functional impacts of the CCHCR1 transgene in mice. This should also be remembered when considering the possible allele-specific effects of the CCHCR1 transgene. In addition, when studying multifactorial diseases, it is always possible that the susceptibility gene alone is not sufficient to produce the phenotype, as additional genes or environmental factors might be needed to trigger the phenotype. One should also perceive the differences in the biology of mouse and human skin. As the functional aspects of CCHCR1 have

remained mainly unknown, the transgenic CCHCR1 mouse offers novel information of the possible role and function of CCHCR1.

As genetic research has failed to convincingly identify the effector gene in the PSORS1 locus, functional studies are needed to elucidate the individual roles of PSORS1 candidate genes and thus facilitate the identification of the PSORS1 gene. Our results support CCHCR1 as an important PSORS1 locus gene, as it is a newly recognized regulator of keratinocyte proliferation, a process severely impaired in psoriasis.

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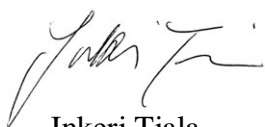
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Espoo, April 2009,

A handwritten signature in black ink, appearing to read 'Inkeri Tiala', with a stylized, flowing script.

Inkeri Tiala

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## **ORIGINAL PUBLICATIONS**